Catalysis and Communication in Dynamic Molecular Networks

Hugo Fanlo Virgós

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Contents

Chapter 1

Dynamic Combinatorial Chemistry as foundation of Systems Chemistry

1.1 Introduction

The gap between chemistry and biology, though apparently modest, seems to be difficult to bridge in practice. The multiple and not fully understood molecular interactions between molecules is the first difficulty that chemists face when trying to mimic the behavior of biological systems. A second level of complexity arises when multiple species interact with each other, behaving as a single system. In this way, the interactions between individual species affect the state of the complete system. In this thesis we introduce some chemical systems which, by using dynamic combinatorial chemistry (DCC), achieve a certain level of molecular complexity and interactions within the controlled conditions of an organic chemistry bench. These systems will be explored for the discovery of catalysts and the construction of molecular communication networks. Catalysts are key elements of biochemical transformations as well as essential parts in many current industrial chemical processes. Molecular networks are ubiquitous, yet not well understood and the communication between different molecular systems could give important information on how chemistry builds up biological systems.

1.2 Dynamic combinatorial chemistry

1.2.1 Definitions

Combinatorial chemistry deals with the preparation of a large number of different molecules by combination of a relatively limited number of smaller building blocks, often leading to the formation of structurally related compounds (Figure 1.1). The synthesis can involve sequential steps which will exponentially increase the number of different molecules[1]. A time-effectiveness is often related to the expression “combinatorial chemistry” widely known within the pharmaceutical industry, where the rapid screening of large libraries of different but related compounds is needed. Nevertheless, scientists have found that combinatorial chemistry has also some limitations when searching for new molecules. As a tool based on random synthesis, the outputs of combinatorial libraries are likely to lack specific chirality and/or rigidity, characteristics that are frequently found in pharmaceutical drugs. High activation energy can prevent the

formation of potentially interesting compounds as the synthesis of the combinatorial products is usually kinetically driven.

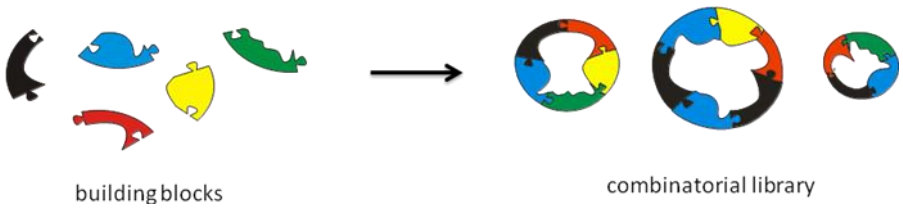


Figure 1.1 Combinatorial library made from different combinations of building blocks. The final composition of the library is fixed and depends on the kinetics of the reactions forming the library members.

Dynamic combinatorial chemistry[2,3] involves the synthesis of relatively complex molecules from simpler molecular building blocks that can react among themselves in a reversible way (Figure 1.2). This means that in a dynamic combinatorial library (DCL) all the products of the mixture can interconvert because of the reversible covalent or non-covalent bonds connecting each of the building units. Following the second law of thermodynamics, the composition of the library will change as the building blocks self-assemble towards the thermodynamic minimum of the system where the product distribution reaches an equilibrium. This equilibrium is a dynamic state: there is a constant formation and disassembly of the products of the library, even though the total composition of the system remains stable over time. Because of the reversibility of the system, kinetic products initially formed due to their low activation energy can be displaced by thermodynamically more stable compounds. In particular cases where the building blocks have two functional groups to reversibly interact with each other, the library is usually dominated by small cyclic compounds (provided the building block concentration is not too high). This composition results in a favorable higher entropy of the system in comparison to the situation where a smaller amount of larger macrocycles would be formed.

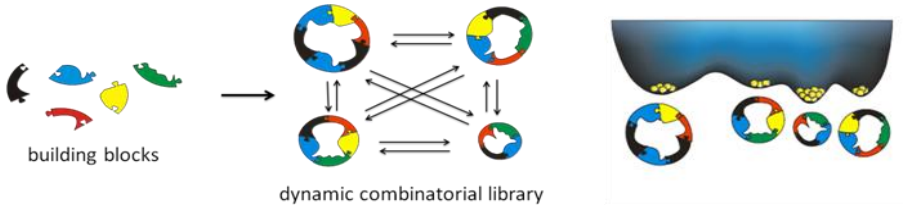


Figure 1.2 A dynamic combinatorial library made from different combinations of building blocks. The library members are constantly exchanging building blocks. The final composition of the library will depend on the thermodynamic stability of the sum of the library members.

1.2.2 Template effects

The state of a dynamic combinatorial library is influenced by thermodynamic variables such as temperature,[4] pressure,[4] or entropy[5,6] of the system. The equilibrium of DCLs can be altered by changing any of these variables so that a new composition becomes the one with the lowest energy. This fact implies that, after a stimulus, the library will reorganize itself by amplifying certain species at the expense of others, shifting its composition (Figure 1.3). For example, when a new species is added to a DCL, interactions between the library members and the new molecules can alter the equilibrium, so the strongest binding molecules will become dominant within the library.[7,8] In this case the composition change is driven by the addition of an external template. It is important to realize though, that the composition of the system does not necessarily include the individually most stable species but rather reflects a combination of species which accounts for the lowest Gibbs energy of the whole system for the particular set of conditions of the experiment.[7-9] Templating effects may also arise from internal changes in the library, leading to new interactions among the library members and resulting in foldamers[10] (covalent oligomers adopting secondary structures stabilized by noncovalent interactions) or fiber-like self-assemblies[11] (noncovalent polymers) that may give rise to fascinating self-replicating species.[12,13] The ability evidenced by DCLs to be templated is the basis of most of the research work presently carried out in the area of DCC.

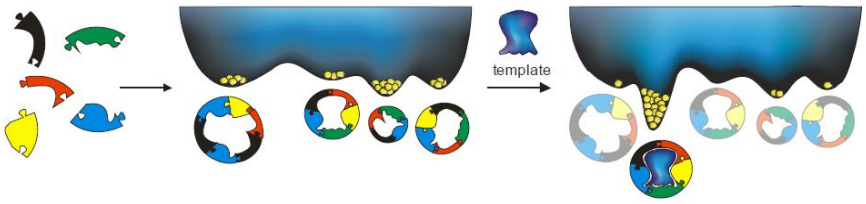


Figure 1.3 A dynamic combinatorial library will reequilibrate upon addition of a template able to interact with the library members.

1.2.3 Design of dynamic combinatorial libraries

When designing a dynamic combinatorial library, the first step is choosing what type of chemistry will be involved in the formation of the reversible bonds which will build the library members. From this selection, a number of variables may become fixed such as solvent, pH, temperature or chemical species compatible with the library. The rest of the building block structure and chemical functionality should be designed in a way that it addresses the purpose for which the library is prepared. In this regard, hydrogen-bond donors and acceptors are valuable moieties for chemical interactions in libraries in organic solvents while for aqueous libraries, motifs leading to hydrophobic interactions are often preferred. In both cases, charged and aromatic building blocks are useful because of their possibility to generate, respectively, Coulomb interactions and π-π interactions.

Reversible chemistries

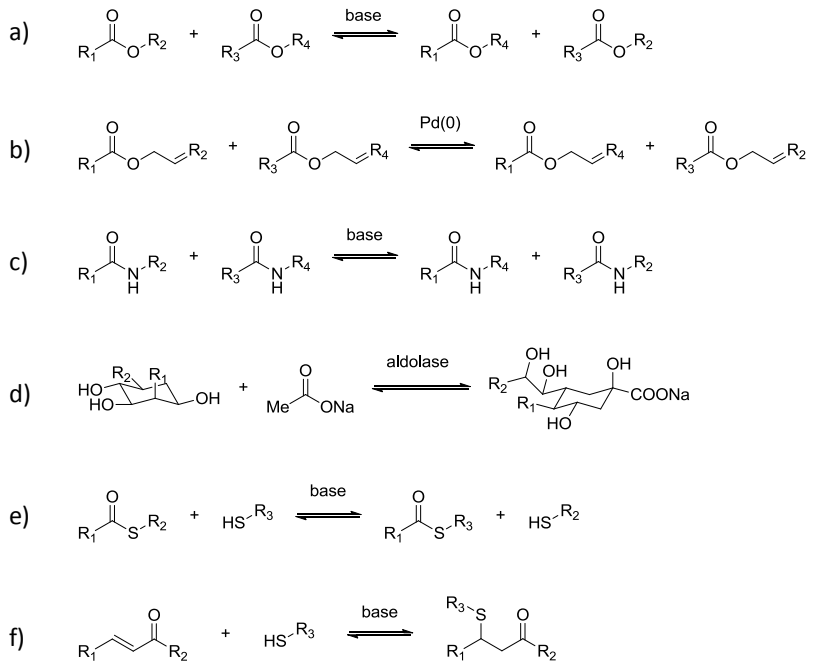
As previously mentioned, in order to obtain a dynamic combinatorial library, the bonds formed between molecular building blocks must be reversible. In this way, the connections can be reverted or exchanged allowing for the generation of different combinatorial products. Some examples using noncovalent interactions such as hydrogen bonding[14-18] and metal-ligand coordination have been already reported.[19-24] However, many applications (see section 1.2.5) require the isolation of the library products, which can be difficult due to the weakness of these types of linkages. To circumvent this problem, an approach using reversible covalent reactions can be used. Acidic or basic conditions, or the presence of metal catalysts are required to achieve most of the reversible reactions used in DCC which are summarized in Scheme 1.1.

Hydrazone exchange and disulfide exchange are the chemistries involved in the work described in this thesis; hence they will be explained in detail in following sections.

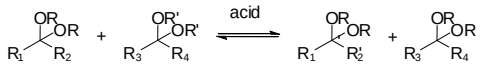
Hydrazone libraries

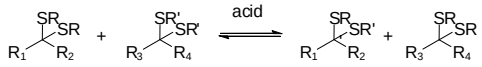
Hydrazone exchange is a widely used reversible reaction in DCC. It involves an initial step of formation of a hydrazone by condensation of a hydrazide and an aldehyde, and a second step where the hydrazone undergoes a nucleophilic substitution by attack of a free hydrazide to the carbonylic carbon from the aldehyde moiety (Scheme 1.2). These reactions can efficiently work in pH ranging from 2.5 to 6.0 as they are favored by the protonation of the aldehyde and the Nitrogen atom in the C=N bond of the hydrazone. Lower pH values will inhibit the nucleophilic attack by protonation of the hydrazide, while at neutral conditions the lack of acidic catalysis retards the hydrazone formation and exchange. Alternatively, nucleophilic catalysts like aniline can accelerate the equilibration of a library at neutral pH and building block concentration ranging between 0.1 and 1.0 mM, decreasing the equilibration time from several days to a few hours.[25,26]

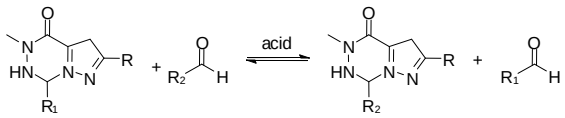
Acyl transfer and related



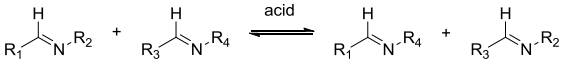
Acetal exchange and related

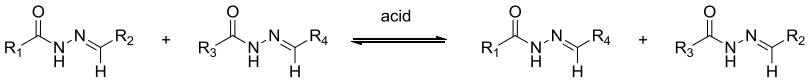
g) 

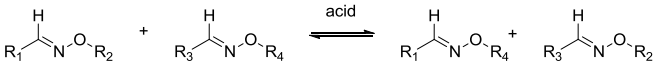
h) 

i) 

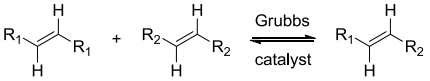
C=N exchange

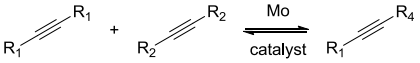
j) 

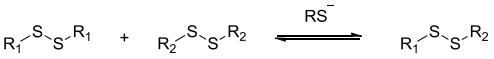
k) 

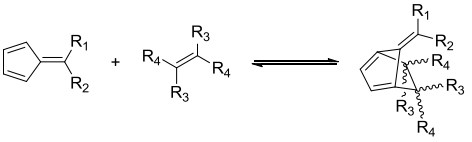
l) 

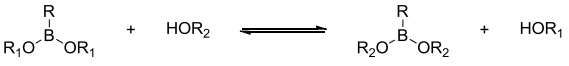
Other reversible covalent bonds

m) 

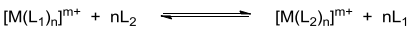
n) 

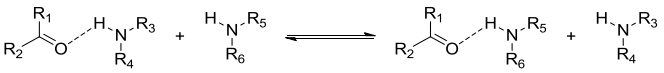
o) 

p) 

q) 

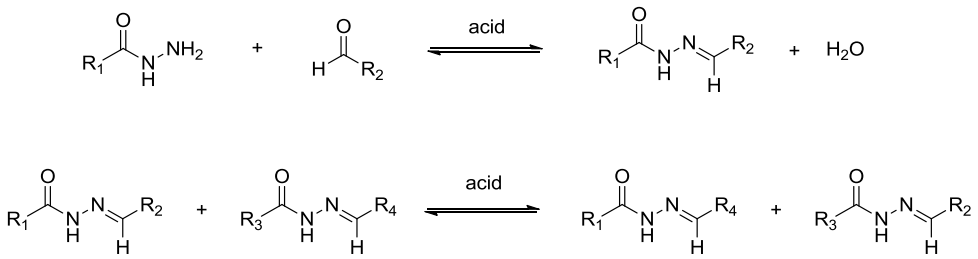
Non-covalent bonds

r) 

s) 

Scheme 1.1 Some reversible reactions used for dynamic combinatorial chemistry: a) transesterification; b) transallylesterification; c) transamidation; d) aldol exchange; e) transthioesterification; f) Michael / retro-Michael reactions; g) acetal exchange; h) thioacetal exchange; i) pirazolotriazone metathesis; j) transimination; k) Hydrazone exchange; l) oxime exchange; m) alkene metathesis; n) alkyne metathesis; o) disulfide exchange; p) Diels-Alder / retro-Diels-Alder reactions; q) boronate ester exchange; r) metal-ligand exchange; s) hydrogen bond exchange.

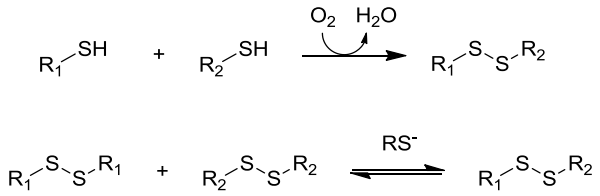
Hydrazone groups are relatively stable in aqueous media as opposed to other similar functional groups like imines, and that is the reason why they are more frequently used in DCC. This extra stability is due to the lower electronegativity of the N atom of the hydrazide compared to the one of the amine.[27] This difference makes the C=N bond less susceptible to hydrolysis by nucleophilic attack of water. As a result, the typical hydrazone equilibrium provides an approximate 90/10 to 99/1 ratio of hydrazone/hydrazide[28] when both reagents are added in equimolar concentrations. This ratio allows for an excellent product yield which benefits the synthesis and analysis of the library members while still keeping a high enough hydrazide concentration to achieve a high reequilibration rate of the library. The addition of an excess of hydrazide may help to accelerate library equilibration.[29]



Scheme 1.2 Hydrazone formation and exchange.

Disulfide libraries

Disulfide exchange is also commonly used in the preparation of DCLs. Thiols are normally employed as starting material. In this case, an initial step of oxidation is needed so that part of the thiol groups in the solution become oxidized to disulfides (Scheme 1.3). The reaction proceeds readily in presence of atmospheric oxygen. The disulfides hence formed, may now exchange by simple nucleophilic attack by the free remaining thiolates in solution. For both steps (oxidation and exchange) to happen, a neutral to mildly basic pH (usually between 7 and 9) is typically used so that thiolates are available in solution.



Scheme 1.3 Mechanism of disulfide formation and disulfide exchange.

An excess of molecular oxygen available to the library may result in a complete oxidation of the thiols which are required for the exchange, therefore bringing the library into a "frozen" state. At this point, the library is no longer able to shift its disulfide composition to achieve a thermodynamic minimum, since it is not anymore a dynamic system. Consequently, it is important to be able to determine whether the final composition of the library corresponds to the one at equilibrium, or if, on the contrary, the library was fully oxidized before it could reach equilibrium. To be able to tackle these issues, the kinetics of the library reequilibration should be fast compared to the oxidation rate of the building blocks. Alternatively, one can "artificially" keep the library in a dynamic state by periodically adding extra dithiol building blocks or some agent able to reduce part of the disulfides back into thiols. An original approach was established during this research work by keeping the libraries under a nitrogen atmosphere to keep the concentration of the oxidized species at a constant level. Working under these conditions, disulfide systems have proven to be dynamic for periods of time longer than 2 months.

1.2.4 Analysis of libraries

One of the advantages of DCC is the possibility of easily obtaining a large range of compounds which are closely related in terms of molecular reactivity and structure. Unfortunately, this advantage turns into a difficulty at the point of analyzing the library composition. Given that screening DCLs for targeted molecular binders requires the ability to measure any increase in the concentration of any of the library members, an ideal analytical technique would include high sensitivity as well as dynamic range. If these two prerequisites are fulfilled, low and high concentrations of any library member can be quantified. With the exception of small libraries containing simple building blocks, NMR spectra of the complete libraries become difficult to interpret due to the overlap of signals. The combination of two techniques which allow the separation and later identification of the mixture of compounds has proven to be the most general solution to analyze DCLs, in particular liquid chromatography-mass spectrometry (LC-MS) (Figure 1.4). The initial separation of the species by HPLC can give information about how many different molecules are present in the library and the relative amount of each of them. Care should be taken though, not to directly associate signal intensity to compound concentration when working with molecules that exhibit different molar absorptivities (ε). The subsequent analysis of the liquid chromatography output by MS should lead to the identification of each of the detected species. In most of the cases, the value of the mass can unequivocally determine the structure of the species investigated.

When different sequence isomers are possible, the analysis of their fragmentation pattern by MS-MS might be required to identify them.[9] Alternatively, nuclear magnetic resonance (NMR) might be a useful technique to fully assign the structure of the library members. The stability of the samples to the LC-MS conditions should of course be tested in advance. For a proper analysis, libraries should be static on the time scale of the analysis, which can normally range from a few minutes to around one hour. In certain cases it is convenient to freeze the library equilibrium prior to its analysis. This can be done by e.g. changing the pH of the solution or by reacting the library members to inhibit their reversible bonds from reacting.

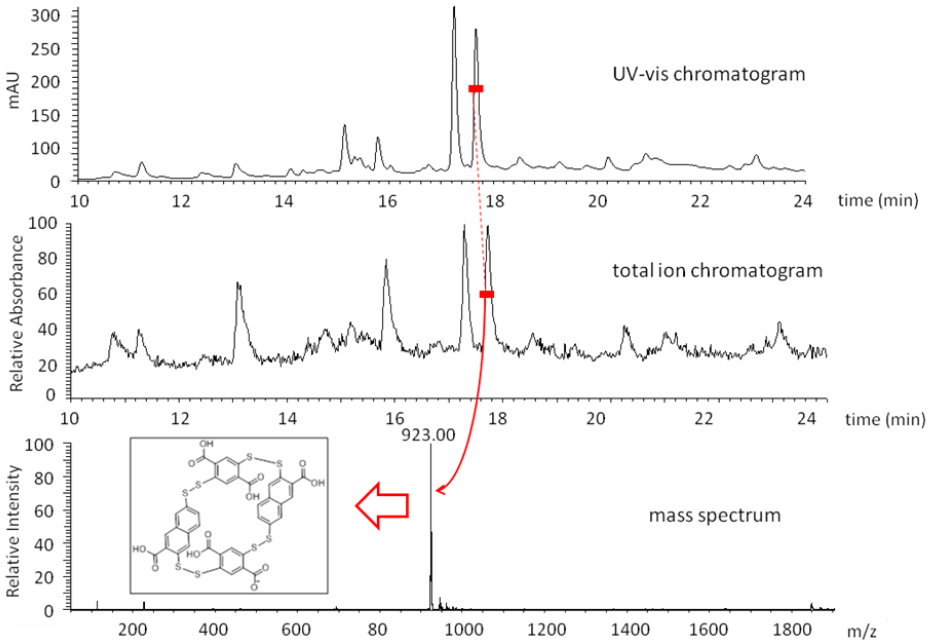


Figure 1.4 UV-vis and total ion chromatogram of a DCL sample and the mass spectrum corresponding to a selected peak.

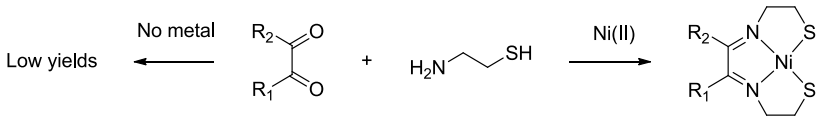
1.2.5 Applications of dynamic combinatorial chemistry

The responsive behavior of DCLs is displayed when the equilibrium of a library is shifted by addition of a templating molecule. This responsiveness is the main advantage of a dynamic combinatorial library over a non-dynamic one, particularly when exploring a recognition process. Examples of uses of DCLs include directed synthesis, discovery of synthetic receptors or sensors.

Directed synthesis

Equilibrium reactions can be tuned towards product formation by following *Le Chatelier's principle* through removing the products from the reaction mixture, adding excess of reagents or controlling the temperature and the pressure. The system will counteract the changes applied on it to reach a new minimum energy state by producing more of the desired product. In a dynamic combinatorial library a template may be added to shift the library composition towards the formation of a selected member of the network.[30] This

synthetic approach is specially interesting when the formation of complex supramolecular structures such as catenanes[31,32] or rotaxanes[33] is required. One of the earliest examples of template-directed synthesis in dynamic systems was achieved by Busch using Ni(II) to synthesize a bis-imino macrocycle (Scheme 1.4).[34]



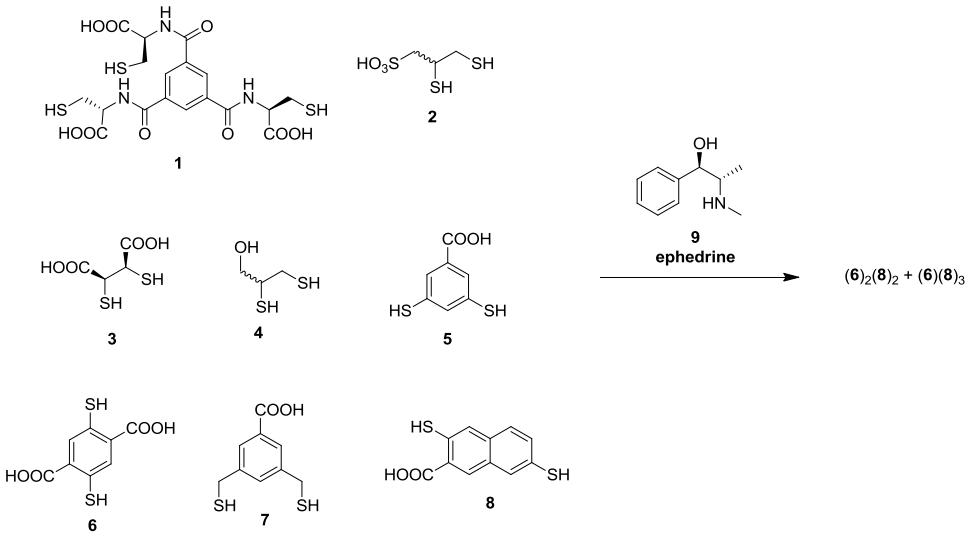
Scheme 1.4 Synthesis of a bis-imino macrocycle directed by Ni(II) templation.

Receptor discovery / Molecular recognition

Dynamic combinatorial libraries can be of enormous utility to discover new synthetic receptors for target molecules. The main advantage in using DCLs is that the synthesis and binding analysis of a high number of the potential binders take place within the same medium without the need for isolating any of them. The possible receptors are self-assembled in the library from simple initial building blocks that were selected to contain functional groups complementary to the ones existing in the target molecule. The interactions between the template and the products of the library will select and amplify the preferred library members which are, in principle, the ones binding more strongly.[7-9] Once the best receptors are detected, they can be either isolated from the library or resynthesized by traditional synthetic methods.

Alternatively, a new combinatorial library containing a selection of the building blocks participating in the formation of the amplified binder can be set up. In this way the assembly of the desired ligand will be further amplified. A significant example of this strategy to discover new receptors was accomplished in our group by identifying ephedrine binders from a dynamic combinatorial library of about 10,000 components (Scheme 1.5).[35]

Aside from biomolecule binders,[36-40] DCLs prepared for the binding of anions[41,42] and cations[43-46] have also been reported.



Scheme 1.5 The presence of ephedrine **9** in a library made from building blocks **1**-**8** amplifies mainly two tetrameric structures from a set of about 10000 possible compounds.

Sensors

Probably the most recent application of dynamic combinatorial libraries is for analytical purposes. The concentrations of the library members in a DCL can be converted into an output signal which varies depending on different factors such as pH, concentration of target molecules, etc. Although generally HPLC is the most common method to analyze DCLs, for sensor applications it is more convenient to make use of faster methods such as fluorescence,[47,48] or UV-vis[49,50] spectroscopy. The spectrum obtained will be a reflection of the analyte interacting with the library. The information about the analyte is distributed along the spectral range of the library members. For this task, the libraries can contain building blocks with dyes covalently attached. Alternatively, the library members can interact through non-covalent bonding with UV-vis active molecules added to the reaction mixture. The presence of a specific molecule able to interact with the same library members can trigger a spectroscopic signal by displacing the UV-vis active molecules out of the library member – dye complex. A clear example by the group of Severin[51] to illustrate this technique employs a set of three different dyes in combination with $CuCl_2$ and $NiCl_2$ to form an initial mixture of complexes. The addition of a dipeptide results in a

release of the dyes which gives a UV-vis signal. The library gives different signals depending on the amino-acid sequence of the added aminoacids (Figure 1.5).

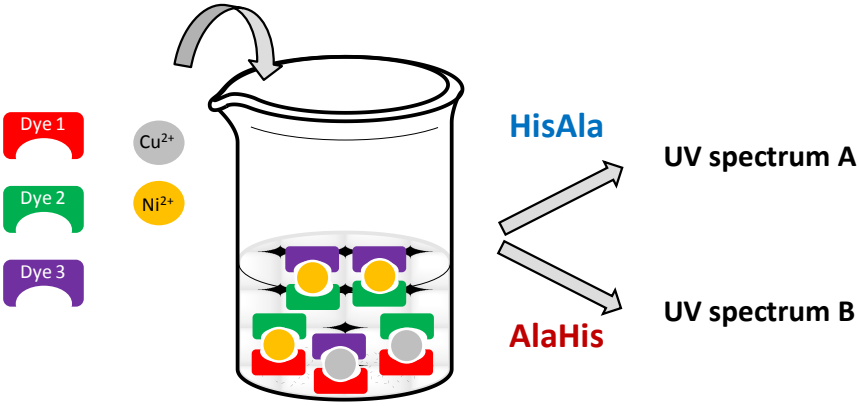


Figure 1.5 A dynamic combinatorial library of dye-metal-dye complexes gives a specific UV-vis spectrum depending on the amino-acid sequence of dipeptides interacting with the library.

Another remarkable example is represented by the chirality sensing of secondary alcohols achieved by Anslyn[52] by analyzing the Zn(II)-ligand self-assembled complexes by circular dichroism (CD).

1.3 Catalysis

1.3.1 Principles of catalysis

A catalyst is a substance able to increase the rate of a reaction by participating in the chemical transformation of the substrates into products. It works by lowering the activation energy (E_a) of a reaction. The presence of a catalyst promotes the formation of a new transition state lower in energy and therefore easier to reach. In consequence, the reaction proceeds faster (Figure 1.6). The catalyst is not consumed in the reaction, although frequently its activity can be reduced by inhibitors which may be produced in the reaction. The opposite case is also possible: a substance can be able to enhance the catalytic activity and in this case it is called catalytic promoter.

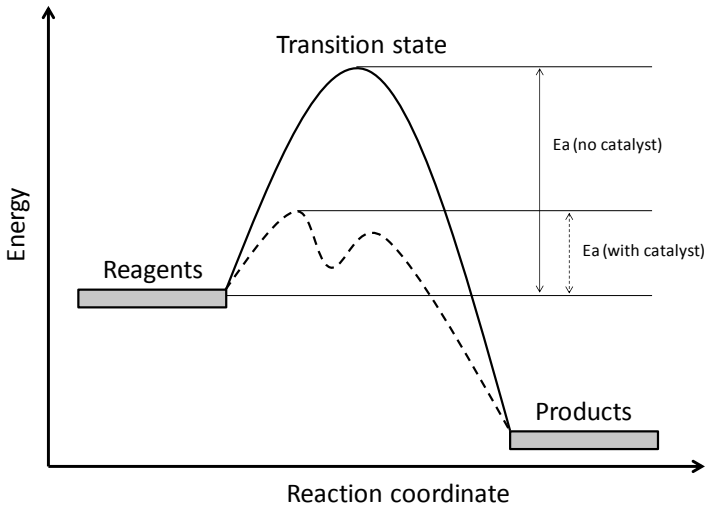


Figure 1.6 Graphical representation of a generic exothermic reaction pathway with and without catalyst. The presence of a catalyst opens a new reaction pathway with a lower transition-state energy while the energies of the initial and final state remain the same.

1.3.2 Types of catalysis

Traditionally, a clear division between homogeneous and heterogeneous catalysis is made (Figure 1.7). While in homogeneous catalysis the catalyst is in the same phase as the reagents (typically dissolved in a solvent) in heterogeneous catalysis the catalyst is in a different phase (normally, a solid in a solution of reagents). In the latter case, the properties of the surface of the catalyst become very important as the reagents will interact only with this surface. In many cases, to improve the surface properties (i.e. increase the surface area) the catalyst is supported on a specific solid.

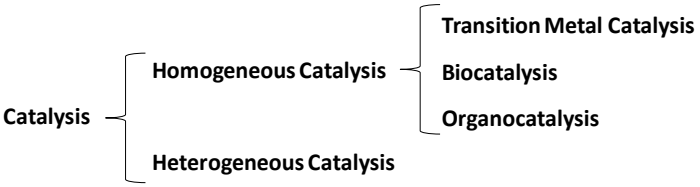


Figure 1.7 Classification of catalysis

Homogeneous catalysis can further be divided in organocatalysis (no metals are employed) or metal catalysis. Transition metals are widely employed as catalysts in small scale and industrial processes. Organocatalysis is less developed than transition-metal

catalysis; however, many of the enzymatic processes lack metal ions and can therefore be considered as organocatalysts, although enzymes are more commonly referred as biocatalysts.

Catalysts can work via covalent interactions with the substrate (DMAP in esterifications) or through non-covalent interactions (hydrogen bonding, hydrophobic interactions, etc.). Mainly the latter are used in supramolecular catalysis.

1.3.3 Supramolecular catalysts

A supramolecular catalyst[53,54] consists of a molecular structure able to bind a substrate through non-covalent interactions to increase the rate of its transformation into the product(s). Supramolecular catalysts, although usually considered as organocatalysts, can sometimes contain metal ions in their structure.

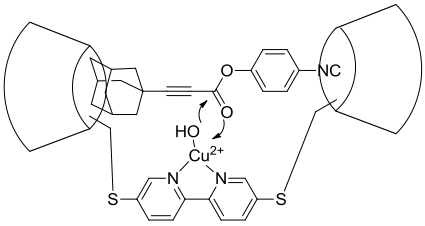


Figure 1.8 Breslow's supramolecular catalyst based on hydrophobic binding interactions with the substrate and nucleophilic attack of a water molecule activated by a metal.

Metals can actively participate in the creation of a new reaction pathway by interacting with the substrate [55-58] (oxidative addition, transmetallation, reductive elimination, etc.) (Figure 1.8) or they can simply act as scaffolds to give shape to the catalysts. In the latter case, the host is assembled by metal-ligand interactions. In a representative example of supramolecular catalysis by Fujita,[59] a cage made from *cis*-capped Pd(II) and a triazine-cored tridentate ligand was able to catalyze a Diels-Alder reaction (Figure 1.9). In the presence of 10 mol% of the host, the conversion was complete after 5 hours, while only 3% of conversion was achieved in the absence of catalyst.

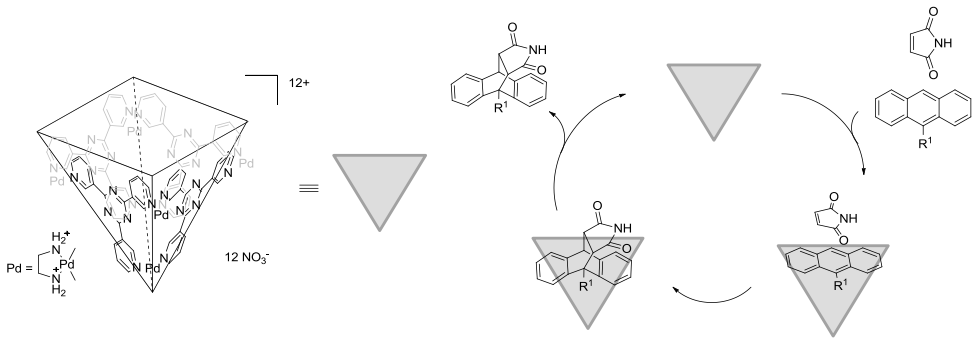


Figure 1.9 Fujita's catalyst structure and catalytic cycle for a Diels-Alder reaction of anthracene and maleimide derivatives.

A common feature shared by supramolecular catalysts is the presence of non-covalent interactions between the catalyst and both the substrate and transition state of the reaction.[60] The supramolecular structure acts as a host for the substrate which is converted into the product within the cavity of the receptor.

Raymond and co-workers were able to accelerate the aza-Cope rearrangement of an enammonium cation up to three orders of magnitude by using a self-assembled metal-ligand host in aqueous solvent (Figure 1.10).[61,62] The hydrophobic cavity created by the supramolecular assembly interacted with the substrate via hydrophobic and cation-π interactions. The restriction of the space in the cavity favors the encapsulation of the tightly packed chairlike conformation of the TS of the aza-Cope rearrangement.

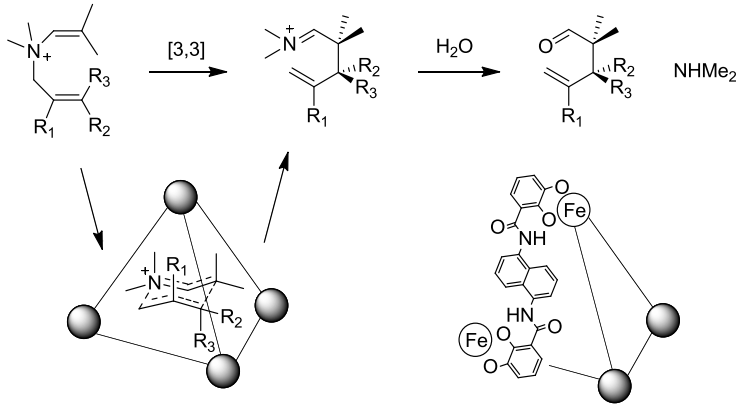


Figure 1.10 Aza-Cope rearrangement catalyzed by a tetrahedral metal-ligand assembly. The subsequent hydrolysis of the product of the rearrangement prevents the catalyst from being bound to the iminium ion, allowing catalytic turnover.

Following Pauling's concepts,[63] the active site of the catalyst is designed to be complementary to the shape and electronic features of the transition state in analogy to the working principles of enzymes. Nature defines very precisely the environment of a catalyzed chemical transformation by selective fitting of enzyme to substrate and transition state. The goal of designing and synthesizing systems of comparable complementarity seems, however, hard to achieve for the traditional synthetic chemist. For this reason, self-assembling systems such as DCLs, where chemical structures can be influenced by the presence of templating agents, can be regarded as a shortcut to prepare complex structures with potential catalytic properties.

1.3.4 Dynamic combinatorial approach to catalysis

Dynamic combinatorial libraries allow for synthesizing and screening for molecular complementarity in one step. This aspect renders DCLs as potentially valuable systems to discover new catalysts. Based on the trial-and-error approach, DCLs may allow a self-adjusting formation of a catalyst which matches the chemical characteristics of the substrate. A catalyst should of course bind to the substrate in order to perform any chemical transformation. But another crucial step is the lowering of the energy of the transition state of the reaction. A good adjustment between substrate stabilization (which occurs if there is preferential binding by the catalyst) and transition state stabilization must be reached in order to obtain catalytic behavior from a dynamic combinatorial library member. It is impossible to know *a priori* if a good binder of the substrate will also be a good catalyst for its transformation into the product. A good substrate binder may effectively stabilize the substrate but not the transition state. On the other hand, good catalysts could transform the substrate within the library environment even if their concentration is relatively low compared to other library members. In this case a good catalyst would remain undetected due to lack of (or very small) amplification. To address this problem, transition-state analogs (TSA) are used to detect library members able to stabilize the transition state of the reaction.[64] This idea is based on the catalytic antibody approach for the formation of supramolecular catalysts. It was firstly suggested by Pauling in 1948[65] and later developed by Jencks, proposing a transition-state analog as antigen to produce catalytic antibodies.[66] If the developed antibodies are able to bind and stabilize

the TSA mimicking the real TS of a reaction, catalysis would be achieved. It has been estimated that there are around 10^{10} potential antibodies, which makes this a powerful approach for developing new catalysts. Some promising examples of the use of catalytic antibodies have been already developed. For instance catalytic antibodies were obtained for the hydrolytic degradation of cocaine[67] (Figure 1.10) or the oxidation of nicotine.[68]

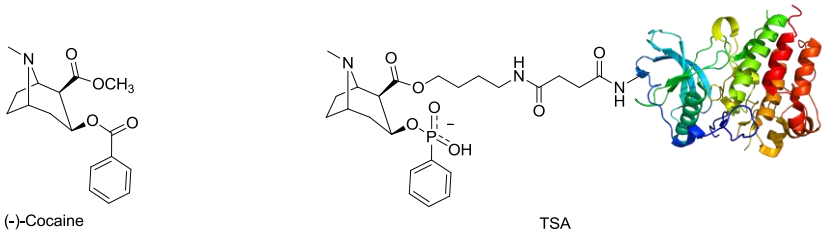


Figure 1.10 Molecule of (-)-cocaine and the TSA used by Landry to obtain catalytic antibodies for the hydrolysis of the benzoate moiety of the cocaine.[67]

In conclusion, when using DCC for discovering catalysts, an important part of the work is done by the system itself. The selection of the preferred functional groups and the spatial configuration that will make a potential catalyst will be determined by the library based on the interactions between catalyst and substrate or transition-state analog. Moreover, the synthesis of the chosen structures will also take place within the dynamic combinatorial library environment.

State of the art. Catalysis and dynamic combinatorial chemistry

To date, very few examples of catalytic applications within DCC have been reported. A dynamic combinatorial library of disulfide molecules was used by our group to identify a catalyst for a Diels-Alder reaction.[64] The product of the reaction itself was used as transition-state analog (TSA) to identify binders of the real transition state (TS) of the Diels-Alder reaction, given the similarity between the TS and the product (Figure 1.11).

Two library members were amplified when the TSA was added to the library. Subsequent isolation of these selected compounds allowed to perform catalysis experiments which showed that one of them was active as a catalyst. An improvement of the reaction rate by a factor of 10 was achieved. The resemblance between TS and product had an undesired consequence: the affinity of the catalyst for the product caused inhibition of the catalysis.

In another example involving DCLs as a source for catalysts, the same disulfide macrocycle was found to accelerate an acetal hydrolysis.[69] In this case, a quaternary ammonium salt was chosen as a TSA to mimic the proposed transition state of the reaction which also contained a positive charge (Figure 1.12). Macrocycle **14** was selected by templating a library of disulfide building blocks. The combination of this preferred cyclic disulfide with the substrate of the reaction showed a modest (factor of 2) acceleration of the rate of the hydrolysis reaction.

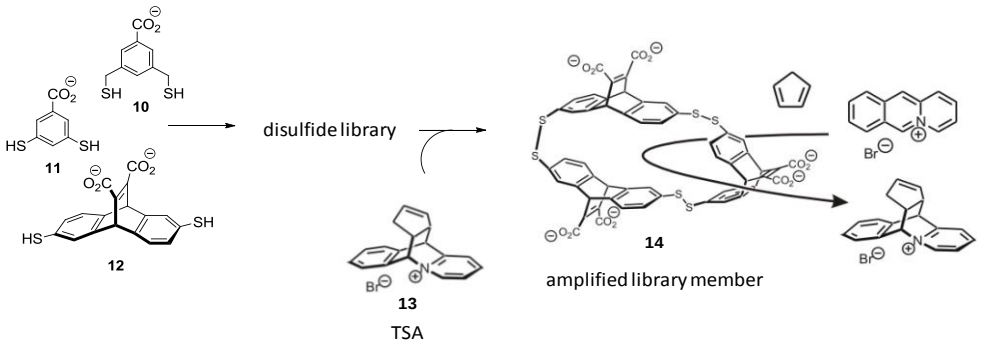


Figure 1.11 A macrocycle is amplified by a TSA of a Diels-Alder reaction in a dynamic combinatorial library made from dithiol building blocks. The selected cyclic homotrimer was found to be catalytically active.

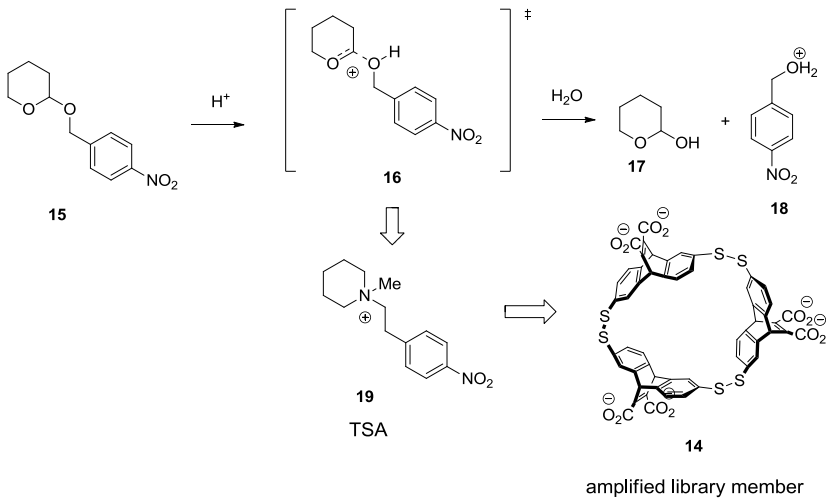
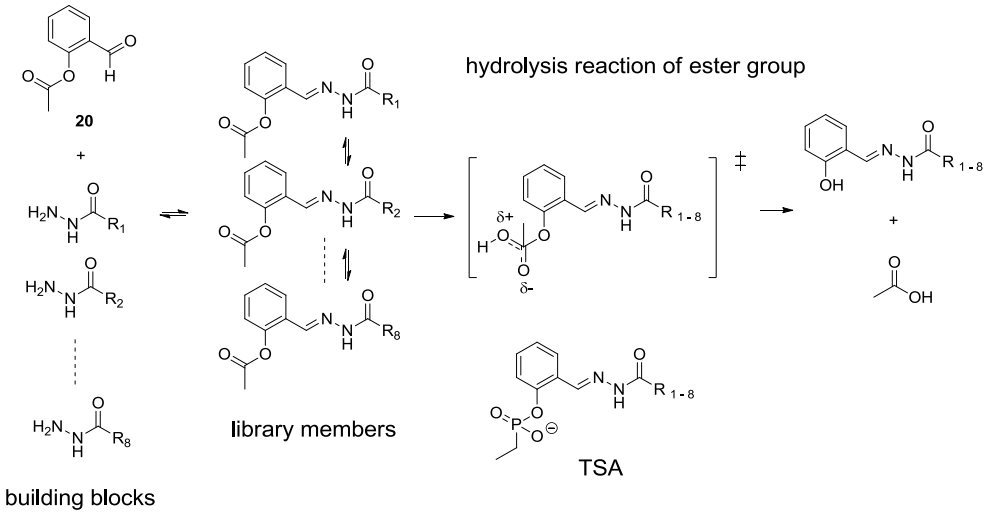


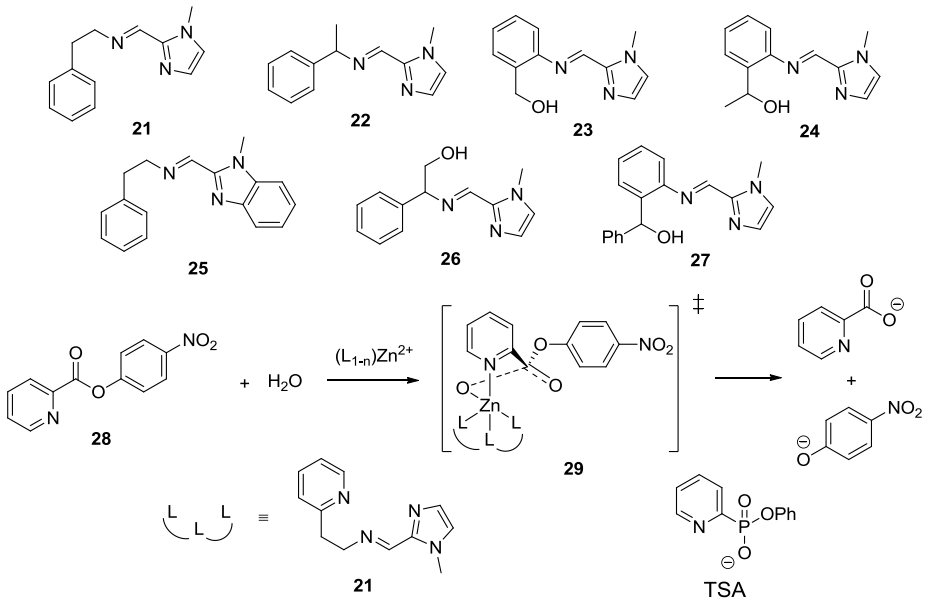
Figure 1.12 Catalyzed acetal hydrolysis reaction and the TSA used to select the catalyst from a disulfide dynamic combinatorial library.

In a different study, Prins and Scrimin compared the catalytic properties of different neighboring functional groups for the hydrolysis of carboxylic esters.[70] Libraries of hydrazones combining a substrate (ester) and different functional groups were tested for differences in rate of hydrolysis of the ester moiety and variations of the rate of reaction up to a factor of 60 were found (see Scheme 1.6). A phosphonate moiety had previously been used as a TSA for the ester hydrolysis. The good correlation between the affinities between the TSA and the selected functional groups and their activity in catalyzing the hydrolysis reaction proved once more the validity of the use of a TSA to identify catalysts.

The group of Nicholas described an approach to develop catalysts from a DCL of imines with the participation of Zn(II) to hydrolyze esters.[71] Adding a TSA, they were able to identify the best combination of amine / aldehyde to achieve catalysis of the hydrolysis reaction (Scheme 1.7). Again, in this example the amplification of a library member within the DCL of imine-Zn(II) complexes of TSAs selected the best imine ligand to stabilize the transition state of the reaction and, consequently, the best catalyst for the reaction.



Scheme 1.6 Scrimin's DCL of hydrazones. The rate of hydrolysis of the ester moieties attached to the hydrazone molecules is influenced by the R substituent. The rate of hydrolysis for each of the library members corresponded well with the concentration of the matching phosphonated TSAs of a similar DCL, proving that the TSAs were a good model for the TSs.



Scheme 1.7 Imine ligands formed in the library and ester hydrolysis catalyzed by a metal-imine ligand complex. The rate of the reaction depends on imine coordinated to the Zn(II). The TSA used identified the imine ligand that promotes catalysis best within a DCL of four different imines.

1.4 Systems behavior of dynamic combinatorial libraries

1.4.1 Introduction

Similarly to how systems biology[72,73] deals with biological networks, systems chemistry can be regarded as an attempt to understand the new behaviors arising from the interaction between simple molecules within complex molecular networks.[74,75] The study of these systems should give a better understanding of how biologically important mechanisms work. This knowledge can ultimately be applied to design new medical therapies or even to engineer new chemical systems able to perform complex tasks that individual chemical entities or simple chemical reactions fail to accomplish. As for now, systems biology is still at the stage of improving the understanding of how the different molecular components interact. Therefore, systems chemistry can be useful at this point by providing some simplified chemical models.

Up to now, most of the applications reported from DCLs were reductionist (section 1.2.5 and 1.3). The different approaches focus on selecting one of the several entities of the system via non-covalent interactions with templating target molecules. The number of

different library members grows exponentially with the different building blocks participating in the DCL. Therefore even a small number of such building blocks can give rise to a relative complex system of interacting species. The study of the behavior of DCLs when considering them as networks of interconnected library members can be regarded as an interesting opportunity to learn about the principles of systems chemistry.

1.4.2 Multiphase dynamic combinatorial libraries

The addition of a new phase (i.e. a second immiscible solvent) to a network of exchanging building blocks can be an advantage from different points of view.[76-80] On one hand, the possibility to increase the number of library members due to the presence of a new solvent able to solubilize additional compounds can lead to an increase in the number of potential receptors for a target molecule. For synthetic purposes, there is a new possibility of shifting the equilibrium of a library towards the compound of interest depending on the solubility preferences of the library members. By tuning the solubility of the building blocks in the different phases, systems with new properties can be created. A careful design of the molecules existing in the system can achieve a new dimension of complexity by limiting the interactions between specific molecules which prefer different solvent phases. This gives the opportunity to simultaneously use certain building blocks that are otherwise incompatible. For example, the group of Sanders has prepared disulfide combinatorial libraries using systems with two phases to overcome solubility problems.[77] Moreover, three-phase systems have been used for the transport of spermine between two aqueous layers through an organic phase.[78] The building blocks dissolved in the organic phase and one of the aqueous phases self-assembled a carrier that allowed polar spermine molecules to cross the apolar phase (Figure 1.13). This work and the report by Lüning on the transport of calcium ions through a bulk phase[79] and membranes[80] are the only examples that make use of DCLs for molecular transport.

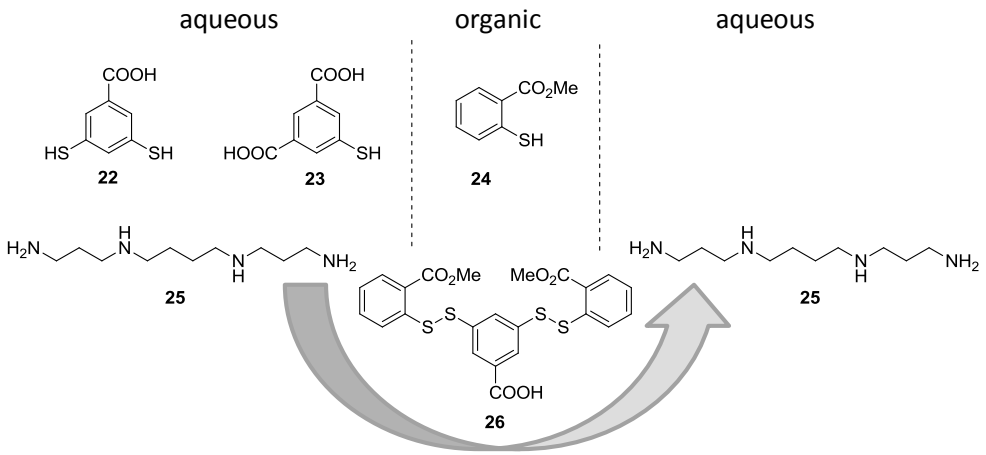


Figure 1.13 A disulfide library in an aqueous-organic system made by building blocks **22**-**24** was templated by spermine (**25**) to amplify the library member **26** which acted as carrier of **25** from one aqueous phase to the other.

An original work on DCC at the interface of a phospholipid bilayer[81] reported that this special environment can lead to a change on the outcome of the dynamic combinatorial library, promoting chain-like compounds over small macrocycles.

1.5 Aims and outline of this thesis

An attempt to bridge the gap between chemistry and biology is described in the following chapters. For that purpose, DCC will serve as a tool to create complex dynamic mixtures from which new exclusive properties can be obtained: from the self-sorting of a catalyst to the communication between systems via chemical signals.

In Chapter 2, an approach to catalysis using hydrazone DCLs containing Zn(II) is developed.

In Chapter 3, an alternative type of DCLs based on disulfide exchange is used to explore metal catalysis.

In Chapter 4, a system able to assemble an organic catalyst in response to the presence of substrate in the reaction mixture is presented. After the substrate reacts completely, the DCL shifts back towards its original composition where the catalyst is a minor species.

Chapter 5 gives an example of how a dynamic combinatorial system can be enriched by having a number of different phases. In particular, the communication between two populations of chemical libraries in different phases has been achieved.

Finally, Chapter 6 presents a summary of the results described in the previous chapters.

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Chapter 2

Cyclen Derivatives in Dynamic Combinatorial Chemistry. Hydrazone Libraries

2.1 Introduction

Traditional metal catalysts in homogeneous catalysis are built from a combination of a transition metal, which acts as a binding site, and a number of ligand groups coordinated to it. The ligands stabilize the metal atom in solution and, if chosen properly, can define the outcome of the catalytic reaction. The formation of such catalysts from simple building blocks is one of the goals in DCC. For creating a supramolecular metal-containing catalyst in a DCL, the metal needs to be held by the ligands to the supramolecular structure. In order for the metal atom to be part of the library members, at least one of the building blocks participating in the library should bind to it. Since cyclic polyamines are known for their ability to bind transition metals,[1-6] 1,4,7,10-tetraazacyclododecane (cyclen) was selected as a promising binding site for metal cations. As an added value, cyclens are also known to bind anions.[7-9] In order for cyclen to be incorporated into DCLs, the original structure was functionalized by attaching hydrazide groups. The resulting building blocks were used to prepare DCLs, first in absence of metal ions, to study their general behavior and the scope of potential guests and/or substrates for catalysis. Afterwards, the compatibility of these DCLs with metals and their behavior was investigated.

2.2 Cyclens in supramolecular chemistry

The chemical properties of 1,4,7,10-tetraazacyclododecane are very much influenced by its specific structural characteristics. Due to the proximity of its NH groups,[10,11] electrostatic repulsions result in differences in the pK_b of the four amino groups. While $pK_{b,1}$ and $pK_{b,2}$ were found to be 10.7 and 9.7 respectively, the values for the third and fourth protonation constants of cyclen drop to $pK_{b,4} < pK_{b,3} \approx 2$.[12] This data explains why at pH values between 2 and 9, cyclen exists mostly as a doubly protonated species. The resulting positive charge promotes the interactions with anions. For example, the recognition of adenosine monophosphate (AMP), diphosphate (ADP) and triphosphate (ATP) by cyclen derivatives was attributed to the combined effect of π-π stacking interactions between aromatic rings and the interactions between the cyclen rings and the phosphate groups (Figure 2.1).[13]

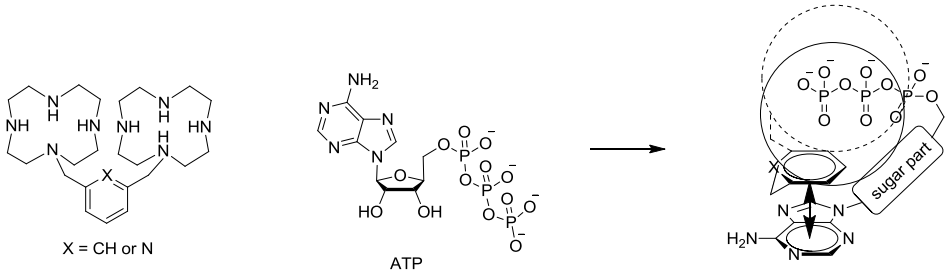


Figure 2.1 Recognition of ATP by a cyclen-based macrocycle.

Much of the research works of cyclen-containing molecules found in the literature exploit the ability of polyamines to bind metal ions. The free electron pairs of the nitrogen atoms can act as coordinating electrons when a transition metal is available. Metal containing cyclen derivatives have not only been extensively used for molecular recognition[14-18] of mostly phosphate derivatives but also for catalysis.[19-21] Examples of metals used for catalytic purposes in cyclen complexes include Zn(II),[4,17,22-25] Co(III),[26,27] Ni(II)[2] and Cu(II)[4] in most cases, although some examples with Fe(III)[28] or heavy metals[29-31] are also known. Among the applications of Zn(II)-cyclen catalysts, perhaps the most widely explored, we mainly[32] find hydrolysis of carboxylate[6,24,25] and phosphate[20,21,33-36] esters. For Co(III)-cyclen complexes, apart from hydrolysis of carboxylate[37] and phosphate[38-42] esters, some cases of peptide hydrolysis[43-47] or nitrile[48] hydration have been described.

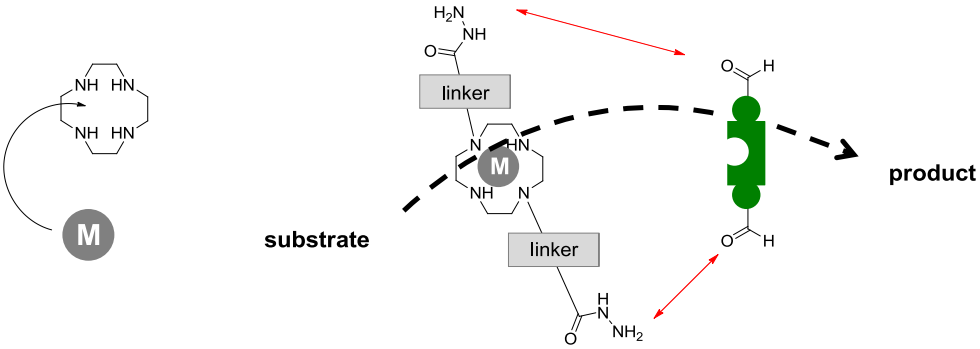


Figure 2.2 The goal: a metal-cyclen building block reacting with a dialdehyde to form a hydrazone which is able to perform catalysis.

An important goal of this work was to form dynamic combinatorial libraries of metal-bearing hydrazones, taking advantage of the ability of cyclen to bind metals. It was

envisaged that a metal-bound cyclen derivative featuring hydrazide moieties would be able to react with dialdehyde compounds to give rise to a set of hydrazone structures that would, in the best case, contain a catalytic species for a given reaction (Figure 2.2).

2.3 Study of cyclen-hydrazone libraries

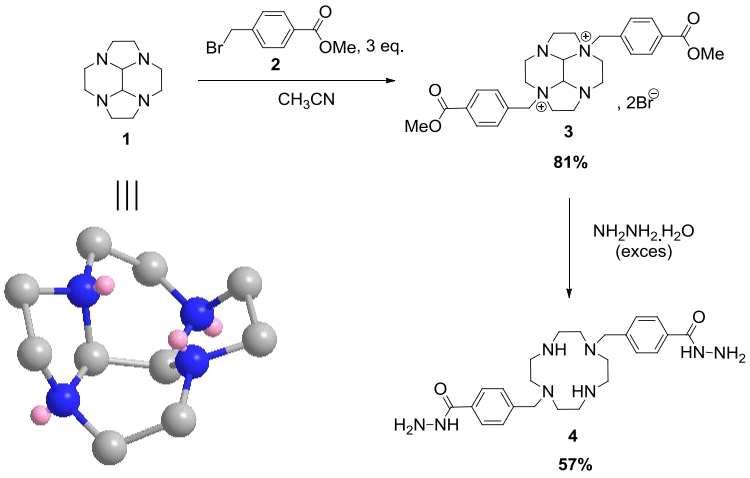
2.3.1 Introduction

Dynamic combinatorial libraries of hydrazone compounds can be prepared by mixing hydrazide building blocks with aldehyde building blocks in acidic aqueous solutions. With host-guest recognition as the desired outcome of our research, structures with a certain rigidity like cycles, spheres or other 3D arrangements may be preferred as they can provide an increased number of interactions with a substrate, thereby allowing for effects of cooperativity. To build cyclic structures in a DCL, at least doubly functionalized molecules need to be used, and in case of DCLs of hydrazones, one possibility is to equip some of the building blocks with two aldehyde groups and the rest with two hydrazide groups. In principle, the cyclen could be incorporated either into the hydrazide or the aldehyde building blocks. Since more dialdehyde compounds were commercially available (compared to dihydrazide compounds), it was preferred to synthesize molecules containing the cyclen ring along with the hydrazide groups.

2.3.2 Synthesis of a cyclen-hydrazone building block

Molecule **4** was synthesized in two steps starting from the commercial glyoxal-protected cyclen **1**.[49] The first step of the synthesis involves a S_N2 reaction between the nitrogen atoms of **1** and the Br substituted carbon of **2**. The substitution proceeds much faster for the two nitrogen atoms of **1** that have their lone electron pairs oriented outwards, than for the other two. This results with the *trans*-benzylated molecule **3** as the dominating product. The reason for this difference in reactivity is the unequal position of the lone electron pairs in the nitrogen atoms of the cyclen. A stronger nucleophilic character is observed for the alternate nitrogen atoms that have electron pairs pointing towards the outside of the cyclic structure.[50]

A final treatment of **3** with an excess of aqueous hydrazine generated the hydrazides by amidation of the ester groups and deprotected the cyclen by reacting with the electrophilic carbons of the glyoxal protecting groups.



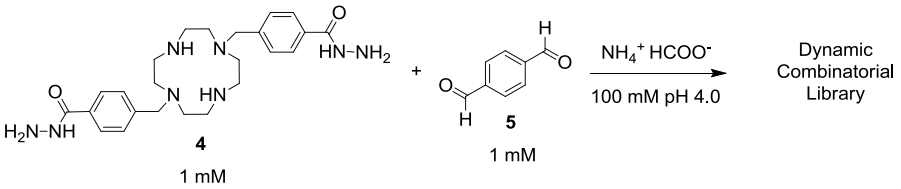
Scheme 2.1 Synthetic route to obtain building block **4**. A ball and stick representation of the structure of **1** (energy minimization by MM2 force field RMS: 0.010) shows two of the lone electron pairs pointing towards the concave part of the cyclen while the other two are pointing outwards.

2.3.3 Preparation of libraries

Most of the host-guest complexes found in aqueous supramolecular chemistry include, to greater or lesser extent, hydrophobic interactions. The presence of these non-covalent interactions allows for the incorporation of an apolar guest into an aqueous solution while avoiding direct contact with the polar water molecules. In addition, the presence of aromatic rings in a host can lead to the formation of π-π stacking interactions and cation-π interactions with the guest molecule, favoring the assembly of the complex. Altogether, the binding of a hydrophobic molecule to a supramolecular host should yield a reduction of the ΔG of the system when compared to the separated substrates.

Since our goal was to achieve the formation of these host-guest complexes, relatively apolar aldehydes were used for the preparation of libraries, many of them included also aromatic rings in their structures.

Initially, for the library preparation a solution of building block **4** in ammonium formate buffer (100 mM, pH = 4.0) was mixed together with a solution of dialdehydes in an organic solvent to reach a final concentration of 1.0 mM of each family of compounds (Scheme 2.2). It was found that, in general, the initial formation of hydrazones took place on a time scale of minutes. The subsequent reequilibration of the initial hydrazones into the thermodynamically stable hydrazones was normally achieved in 3-5 days. Whenever templating effects were explored, the molecule tested for interactions with the library was added to the solution at the same time as the starting building blocks.



Scheme 2.2 Preparation of a typical dynamic combinatorial library from a hydrazide and an aldehyde containing building block.

2.3.4 Optimization of the library conditions

Previous work on aqueous dynamic combinatorial libraries in our group required the use of a variety of buffers (borate, phosphate, ammonium acetate or ammonium formate buffers, among others) to keep the solution at a constant pH. Ammonium formate was a good candidate to buffer the solutions of hydrazones at a pH of around 4, since the pK_a of formic acid is 3.77. This buffer offers an additional advantage as it easily vaporizes when the sample is injected in a mass spectrometer for analysis. It had already been successfully employed in our group for hydrazone exchange and therefore we decided to use it for the cyclen containing libraries in 100 mM concentration and pH = 4.0.

Organic co-solvents are frequently added to achieve a good solubilization of all the compounds in the libraries. The precipitation of a building block or a library member may represent a kinetic trap in the system and therefore it is important to avoid it. For this reason, different co-solvents such as CH_3CN, THF and DMSO were tested in the hydrazone libraries previously described with buffer/organic solvent ratios from 8/2 to 5/5. As no apparent difference in the composition of the libraries was observed, it was concluded that any of the mentioned solvents was suitable to aid in the solubilization of DCLs.

Finally, the concentration of the building blocks was also tuned for the best performance of the libraries. Libraries with concentration of 0.1 mM of hydrazide building block **1** were found to take longer time to equilibrate than 1.0 mM concentration libraries (6-7 days vs. 3-4 days). Concentrations above 2.0 mM led to formation of precipitates and were therefore avoided. In view of these results, it was decided that 1.0 mM concentration of **1** would be used in combination with 0.75 to 1 equivalents of total aldehyde(s) as standard concentration, knowing that a small stoichiometric excess of hydrazide improves the equilibration kinetics.[51]

The presence of aniline in the libraries was also tested as a catalyst for the equilibration of hydrazones. Initially reported by Dawson in 2006,[52] the catalytic properties of aniline within DCLs of hydrazones have been successfully utilized in the preparation of protein-templated hydrazone libraries.[53] In our studies, the equilibration of cyclen-hydrazone libraries was also found to be much faster in presence of aniline. Nonetheless, in many occasions it was preferred to avoid the use of aniline in order to monitor the change of the library composition over time.

2.3.5 Influence of the aldehyde on the hydrazone structure

With the aim of obtaining information about the structures that could be expected from cyclen containing hydrazone libraries, nine dialdehydes were selected to prepare DCLs in combination with dihydrazide **4** (see Figure 2.3). Initially, binary libraries containing only one of the dialdehydes were prepared by mixing building block **4** with the building blocks **5**-**13**. As expected, the initial experiments revealed a preference of the combinatorial libraries to form cyclic structures.

As observed in Figure 2.4, when mixing building block **4** and terephthalaldehyde (**5**), the main compound in the library had a cyclic structure where four hydrazone bonds connected two molecules of **4** and two molecules of **5** in an alternated fashion. Additionally a small amount of linear trimer formed by one molecule of **5** and two of **4** was also found in the library, due to the presence of an excess of **4** in the mixture.

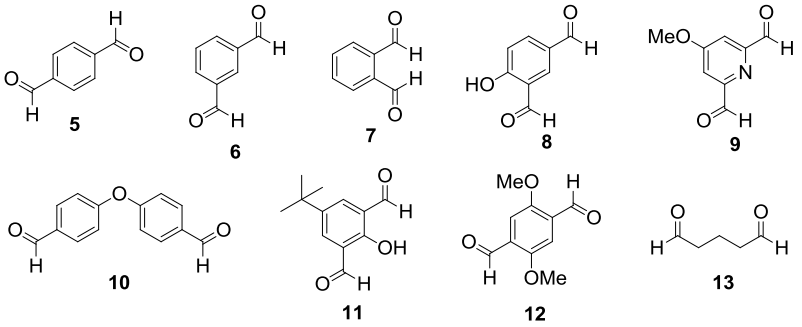


Figure 2.3 Dialdehyde compounds used for the preparation of hydrazone DCLs.

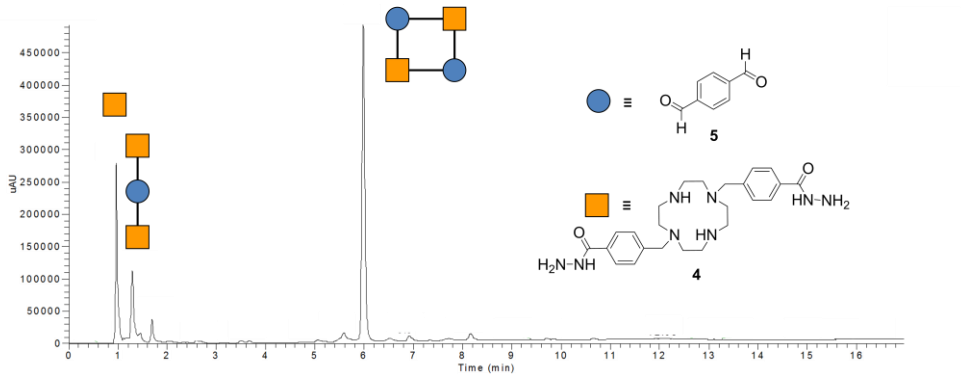


Figure 2.4 HPLC trace of an equilibrated dynamic combinatorial library made from mixing building blocks **4** (1.0 mM, squares) and **5** (0.75 mM, circles) in a 100 mM $NH_4^+HCOO^-$ buffer of pH = 4.0 / acetonitrile (6/4) after 18 days.

Isophthalaldehyde (**6**) has its carbonyl groups in *meta* position, forming a structure with a smaller "bite angle" when compared to terephthalaldehyde. The structural change of the aldehyde had important effects on the composition of the library, resulting in an increased variety of hydrazone products. The analysis by HPLC of a DCL made from **4** and **6** (Figure 2.5) revealed the presence of a cyclic hexamer and a cyclic dimer as well as the cyclic tetramer and linear trimer. The emergence of the cyclic dimer in this library might be explained by the proximity of two aldehyde groups of **6** to two hydrazide groups belonging to a single molecule of **4**, as compared to that in **5**.

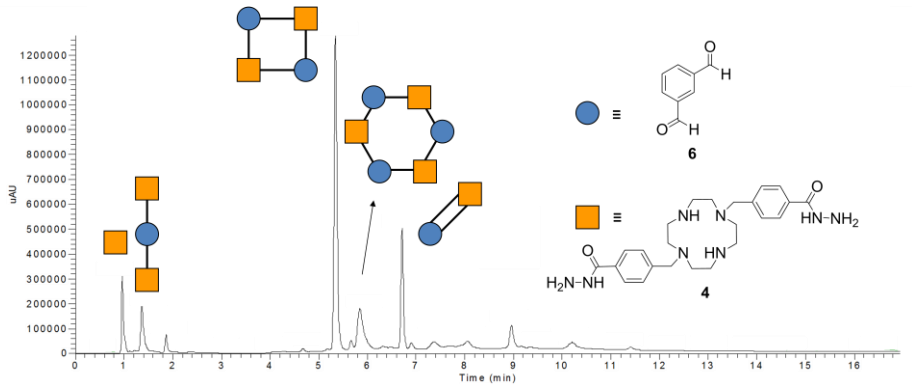


Figure 2.5 HPLC trace of an equilibrated dynamic combinatorial library made from the building blocks **4** (1.0 mM, orange) and **6** (0.75 mM, circles) in 100 mM $NH_4^+HCOO^-$ buffer of pH = 4.0 / acetonitrile (6/4) after 18 days.

The absence of cyclic hexamer in the DCL formed with **5** was difficult to predict. Since the angles in a hexagon are larger than the ones in a square, dialdehyde **5** should easily form hexamers similar to dialdehyde **6**. Nevertheless, so many degrees of freedom in the hydrazone cycles makes difficult to predict which structures will be favored over the others. As suggested by modeling,[54] the assembly of a hexameric hydrazone gives rise to a structure which can fold over itself, as opposed to the dimer and the tetramer (Figure 2.6). At this level of molecular complexity, intramolecular interactions may start to be involved in the stabilization of the structures. Because of that, it becomes difficult to rationalize structural preferences between combinatorial library members.

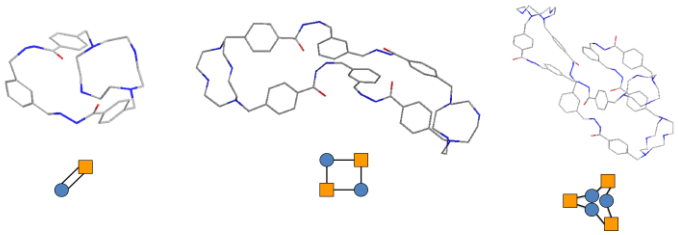


Figure 2.6 Graphical representation of the cyclic dimer, tetramer and hexamer obtained from a DCL of building blocks **4** and **6**.[54]

The third aldehyde of the series, phthalaldehyde (**7**) showed a different behavior as a result of the unusual position of the two carbonyl substituents. The proximity of both aldehyde groups resulted in a chemical reaction with **4** that produced phthalazine as

observed by HPLC (Figure 2.7). Although a cyclic dimer was also found in this library, the presence of hydrazones was otherwise negligible. The use of **7** in DCLs was therefore limited.

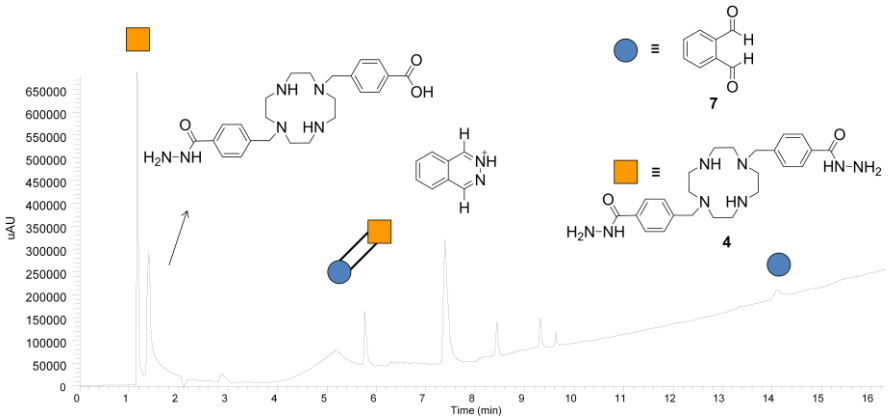


Figure 2.7 HPLC trace of a dynamic combinatorial library made from building blocks **4** (1.0 mM, squares) and **7** (0.75 mM, circles) in 100 mM $NH_4^+HCOO^-$ buffer pH = 4.0 / acetonitrile (6/4) after 18 days.

Compounds **9** and **10** gave rise to libraries where the main hydrazone was the cyclic tetramer and cyclic dimer respectively. The libraries prepared with compound **11** produced a precipitate that could not be dissolved by adding more organic solvent (CH_3CN) and hence were not further investigated. The only aliphatic dialdehyde used, glutaraldehyde (**13**), afforded solely a cyclic dimer when combined with **4** in formate buffer. Thus, it appears that the increased flexibility of **4** in comparison to aromatic dialdehyde compounds of comparable length was responsible for the formation of the smallest macrocycle (Figure 2.8).

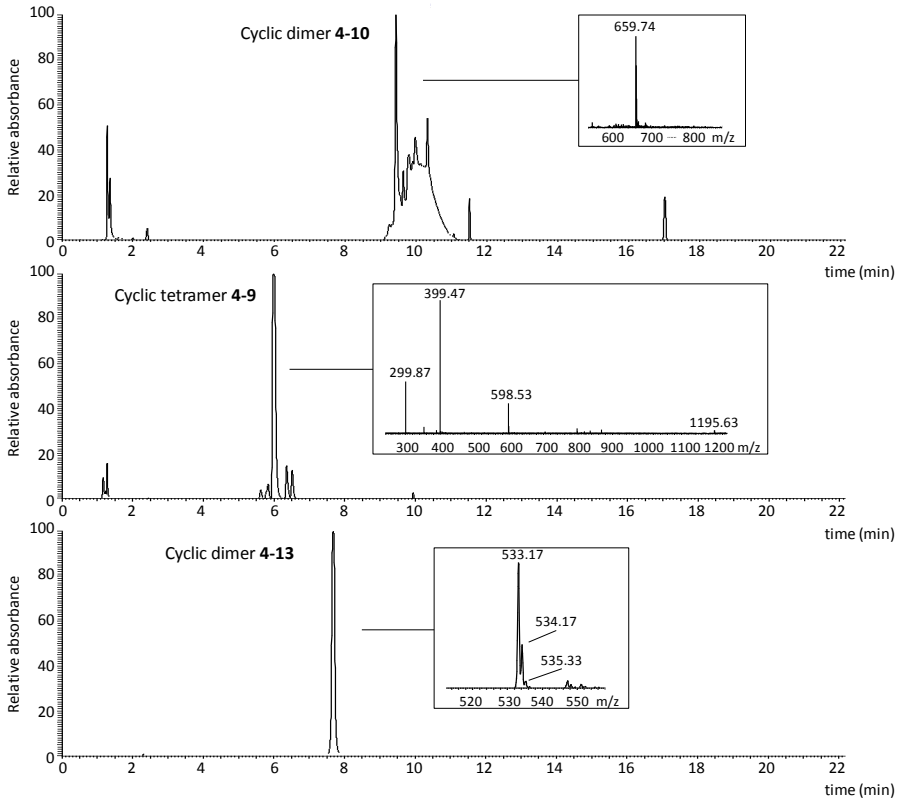


Figure 2.8 LC-MS analysis of hydrazone libraries made by mixing **4** (1.0 mM) with 1 eq of **10** (top), **9** (middle) and **13** (bottom) in 100 mM $NH_4^+HCOO^-$ buffer pH = 4.0 / acetonitrile (8/2) after 10 days.

Libraries containing mixtures of **4** and various dialdehyde compounds were also prepared. Increasing the number of different building blocks mixed together resulted in an increase in the diversity of the library members. The difference in the relative concentration of the library members of equal ring size and made of different dialdehyde compounds was not remarkable, probably due to the similarity of the structures of the aldehydes. For example, a library containing dialdehyde compounds **5** and **6** provided a ratio of cyclic tetramers of 1.000 / 1.424 / 2.256 for the tetramer combinations containing **5**,**6**, **6**,**6** and **5**,**5** respectively (Figure 2.9).

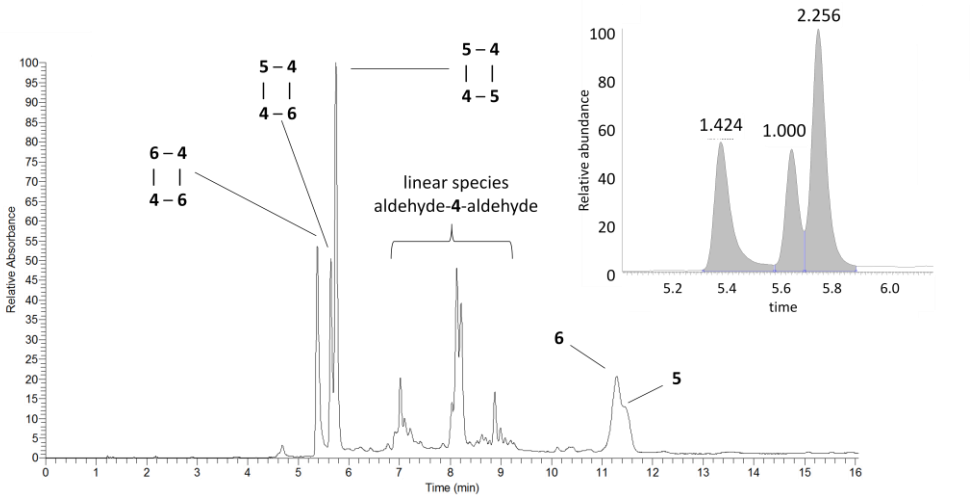


Figure 2.9 HPLC analysis of a library made from hydrazide **4** (1.5 mM) and dialdehydes **5** (1.0 mM) and **6** (1.0 mM) in a mixture $NH_4^+HCOO^-$ buffer 100 mM pH = 4.0 / acetonitrile (6/4) after 8 days. The concentration ratio of the three tetramers is shown in the inset.

The statistical distribution of tetramers would be 2/1/1 with the heterotetramer being the most abundant species in the mixture. The different library distribution obtained experimentally indicates that the structure of the aldehyde has indeed an influence on the stability of the hydrazones. In this particular case, the heterotetramer seems to be thermodynamically less stable when compared to the homotetramers; i.e. the systems has a tendency towards self-sorting.

When mixing several aldehydes with one hydrazide, it may be more advantageous to use a sub-stoichiometric concentration of the hydrazide with respect to the total aldehyde concentration so that the hydrazide will react to form the most stable hydrazones preferentially. In this case, and provided the molar absorption coefficient is equivalent for all the hydrazones, the peak areas of the HPLC traces will be proportional to the amount of each library member, so a good comparison between hydrazone stabilities can be made. In the opposite case, when a binary combinatorial library is prepared with excess (or equimolar amounts) of a non-competing building block (hydrazide, in this specific case), the outcome will probably not reflect the relative stabilities of the library members. This is due to the fact that the building blocks forming the less stable library members will have non-competing building block available to react with and to form hydrazones and

may, depending on the initial composition of the library, even exceed the amounts of the more stable hydrazones. A graphical example is shown in Figure 2.10.

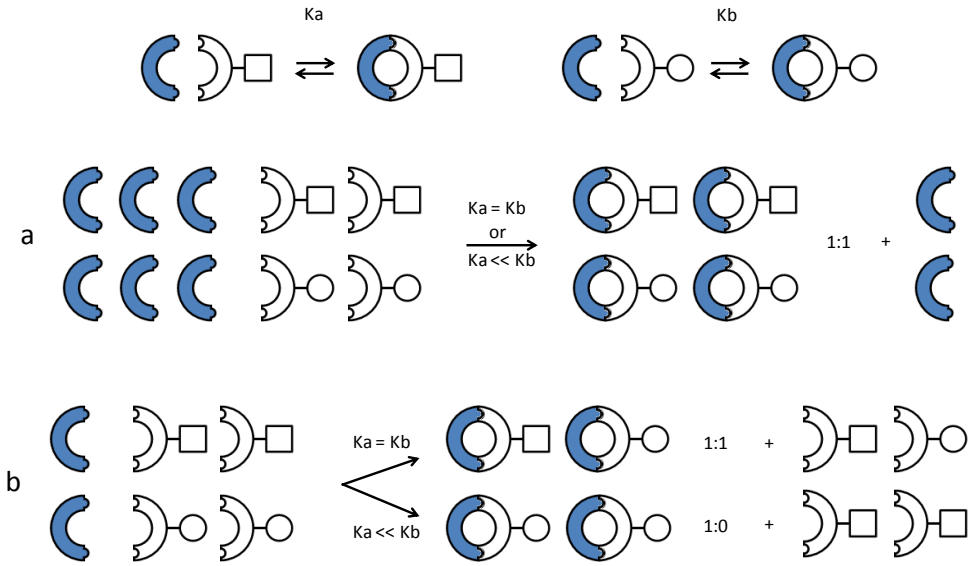


Figure 2.10 Competition between formation of two library members. In a library (a) with an excess of non-competing (colored) building block the final composition will not depend on the relative stability of the library members. In a library (b) where the non-competing building block is present in substoichiometric quantity the library composition at equilibrium will reflect the relative stability of the library members.

These considerations are reminiscent of the ones previously reported by Severin[55,56] and our group[57] regarding the best concentration of template in a DCL that is necessary to achieve a selective amplification of the best binder.

Taking into account the discussions above, systems of higher complexity were studied. For example, a library containing building block **4** and four different dialdehyde compounds (**5**, **9**, **10**, **12**) was prepared. In this case, the concentration of each aldehyde was set to be equal to the concentration of **4**, making the total aldehyde concentration 4-fold of that of the hydrazide concentration. The LC-MS analysis of the library revealed the presence of mainly cyclic tetramers and linear trimers. The library composition can now be considered biased towards the formation of linear trimer, due to the large excess of dialdehyde building block. Nevertheless, these conditions are still beneficial compared to the excess of hydrazide in the system because the library is still highly selective towards the

formation of the most stable hydrazone. From Figure 2.11 it can be observed that the formation of tetramers containing aldehyde **9** is preferred. In contrast, the presence of aldehyde **10** in the hydrazones is negligible.

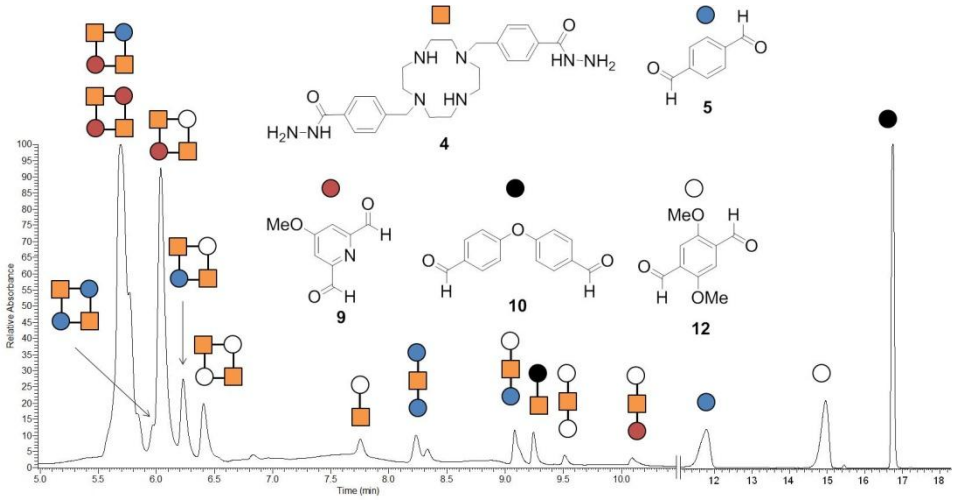


Figure 2.11 HPLC analysis of a library made from hydrazide **4** (1.0 mM) and aldehydes **5**, **9**, **10** and **12** (1.0 mM each) in H_2O (pH adjusted to 5.0 with aqueous HCl) after 5 days. Cyclic tetramers were the most stable species with some linear compounds also being present.

To rationalize this observation, the important role of solvation effects in supramolecular chemistry should be considered. Since **10** is the most hydrophobic aldehyde in the group, the formation of macrocycles containing **10** may be disfavored due to an excessive increase of the energy of hydration. Therefore, it seems to be energetically preferred to have a more soluble macrocycle built from an alternative aldehyde and a less soluble free aldehyde (**10**).

Summing up, a significant variety of structures in the cyclen-hydrazone library can be achieved by properly selecting the structure of the aldehydes. An important issue to take into account is the solubility of the hydrazones. The condensation of hydrazides and aldehydes eliminates polar atoms from the solute, making the products less soluble in water than the starting materials and risking precipitation. Finally, when looking for amplification of receptors in a library, it should always be considered which stoichiometry

of the building blocks is the most appropriate to reach a good correlation between the selectivity of the potential ligand and the amplification of the library members.

2.3.6 Templating assays

The search for synthetic receptors can benefit from dynamic combinatorial chemistry. The host-guest chemistry that is developed when searching for receptors can be well accomplished by DCC that simultaneously produces and tests complete families of potential receptors.

With the aim of detecting stabilizing interactions that could lead to the discovery of hydrazone-containing receptors, a diversity of molecules was added to the hydrazone libraries. Some libraries contained mixtures of **4** with a unique aldehyde as shown in Figure 2.2. More complex libraries were also prepared, containing building block **4** and a larger number of aldehydes. For example, the library containing four aldehydes (**5**, **9**, **10**, **12**) in Figure 2.11 was extensively tested for templating effects. A large variety of potential templates was evaluated. Some of them were biologically important molecules with negative charge aiming to interact with the positively charged cyclen: nicotinic acid, uridine, thymine, thymidine, ATP, ADP, AMP and NaH_2PO_4. Other compounds likely to interact with cyclen such as cyclohexane-1,3-dione, bis(p-nitrophenyl)phosphate, 8-hydroxyquinoline, 2,5-dihydroxypyridine, sodium benzenesulfonate, p-mercaptobenzoic acid and pyrocatechol violet were also tested for binding to hydrazones containing **4**. A large diversity of metal salts such as Cr(III), Mn(II), Fe(III), Co(II), Ni(II), Cu(II), Zn(II) and Gd(III) were added to the libraries as they are known to bind to cyclen and consequently, they could influence the structure of the hydrazone. This association was expected to lead to some selectivity in the assembly of structures within the library.

Unfortunately, no templating effects in the hydrazone libraries could be observed using any of the species mentioned above. It was then decided to make use of metal salts as templating agents, but this time in combination with a variety of coordinating diamino compounds with the aim of increasing the effects of the metal templates by second coordination sphere interactions (e.g. by bridging between two metal coordinating cyclen moieties). For that purpose, a library made of building blocks **4** and **6** was prepared. One equivalent of $Cu(NO_3)_2$ and one equivalent of the corresponding diamine were added

(Figure 2.12). After the analysis of the libraries, it was clear that none of them induced any variation in composition with respect to the library in absence of Cu(II) and diamine and therefore no templating effects were obtained.

The lack of templating response by any of the compounds mentioned above brought up a dilemma about the ability of the hydrazone species to recognize the guests that were added to the system. Two hypotheses were contemplated: the first possibility was that any binding of the template molecules failed to translate into an effect on the library distribution due to the absence of selectivity in the interaction between template and library members. The binding of the template can take place on the face of the cyclen towards the inside of the hydrazone cycle or on the contrary, the binding can occur on the outer face, which would explain a lack of response from the library. The second hypothesis was that the structure of **4**, containing two pendant arms, was preventing any interaction with the template molecules. Either scenario would require a change in the structure of the building block to solve the lack of influence of the template on the library distribution. However, there is still the possibility that the substrate, although not forcing a change in equilibrium distribution, is able to interact with the library members and transformed into the product through a catalytic process. Therefore, it was decided to further investigate the potential of building block **4** in catalysis.

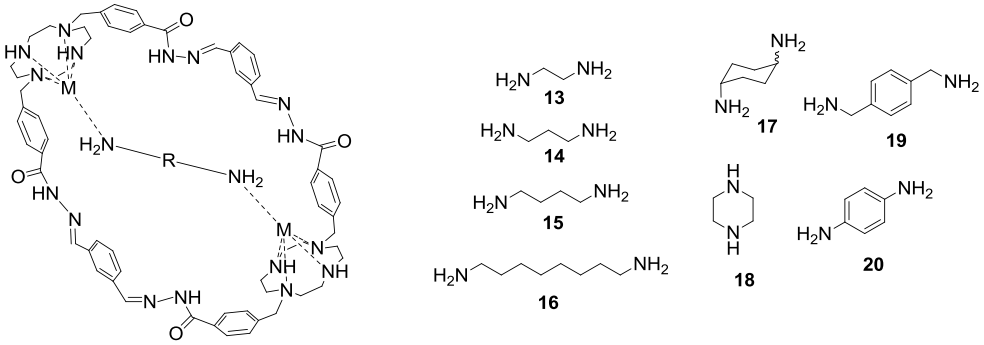


Figure 2.12 Representation of a tetrameric hydrazone where metal ions are coordinated to the cyclen moieties and to a diamino compound (left) and structures of the diamino compounds tested for templating effects in combination with Cu(II) and Zn(II) (right).

2.4 Metal-cyclen complexes for dynamic combinatorial libraries

2.4.1 Formation of cyclen-metal-complexes

Our previous experiments using metal salts as templates for hydrazone libraries did not yield the expected results. In order to obtain information about the binding between metal ions and **4**, titration experiments were performed. ^{1}H-NMR and UV-vis titrations were carried out to compare the interactions of bare cyclen and **4** with Zn(II) and with Cu(II) in order to confirm the capability of **4** to coordinate metal ions. The binding constant of Cu(II) to **4** was obtained via UV-vis titration. The increasing light absorption of the metal complex at 310 nm was monitored and plotted against the concentration of Cu(II). The resulting graph in this way obtained was fitted to a theoretical curve (details in the experimental part) to obtain a value of K_{bind} = 5x10^{5} M^{-1} for building block **4** (Figure 2.13).

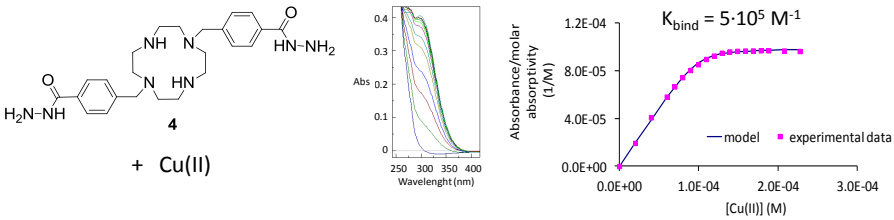


Figure 2.13 UV-vis titration of $CuCl_2$ to compound **4** and the corresponding fitting of complex absorption (310 nm) against added Cu(II) to calculate its binding constant. Conditions: 0.1 mM of **4** in $NH_4^+HCOO^-$ buffer 100 mM pH = 4.0 / acetonitrile (6/4).

The value of the binding constant for cyclen was reported[58,59] to be in the range of K_{bind} = 10^{25} M^{-1} while the value for the 1,7-dimethylated cyclen was found[60] to be K_{bind} = 10^{18} M^{-1}. A decreasing trend of the binding constant with higher substitution of the cyclen was observed. It is well established that tertiary amines are less effective σ-donors than secondary amines.[61] This fact is partially attributed to the lack of hydrogens bound to the nitrogen atoms in tertiary amines, which would help to stabilize the partial positive charge on nitrogen by formation of hydrogen bonds with the solvent. In addition, the presence of the extra N-bound substituent in tertiary amines sterically hinders their coordination to the metal ion.[62]

The binding affinity of Zn(II) to **4** was also studied. In the case of Zn(II), as it is a diamagnetic metal ion, it was possible to monitor the shift of the ^{1}H-NMR signal of the benzylic hydrogens in **4** caused by the addition of the metal. After plotting this variation against the amount of zinc added, it was fitted to a model to extract K_{bind} = 2x10^{4} M^{-1} as the value of the binding constant between **4** and Zn(II) (Figure 2.14).

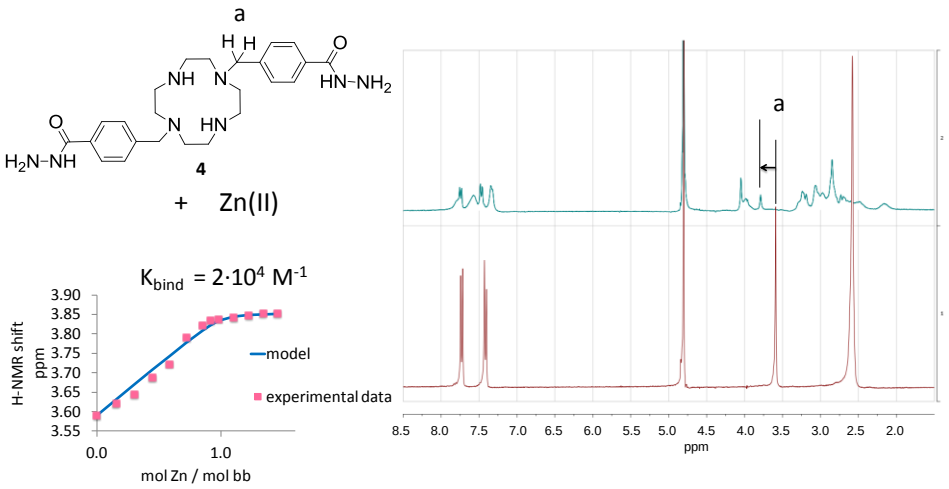


Figure 2.14 ^{1}H-NMR titration of $Zn(NO_3)_2$ to 12.0 mM solution of **4** in D_2O. The shift of signal **a** corresponding to the benzylic hydrogens was monitored and fitted to a 1:1 binding model (see experimental section) to obtain the binding constant.

The titration experiments suggested that Cu(II) and Zn(II) are able to bind building block **4**, but with apparent affinities that are weaker than expected. Furthermore, due to the lack of templating effect upon addition of these metal ions to the libraries, it was decided to synthesize metal complexes of compound **4** in order to prepare metal-hydrazide complex samples to perform further binding studies on the building block. For this purpose, building block **4** and $Zn(NO_3)_2$ were boiled in MeOH to afford **21**. A UV-vis spectrum was recorded and compared to the one of a solution of **4** after adding $Zn(NO_3)_2$, and some clear differences were noticed (Figure 2.15). Kinetic studies performed by Kaden[63] revealed that the formation of metal complexes with macrocyclic polyamine compounds is in fact very slow when compared to the linear polyamines. Altogether, these data led us to believe that only a partial complexation had been achieved when metal salts were simply added to the hydrazone libraries. In order to ensure efficient metal-ion binding, we

decided to direct our efforts to the study of DCLs made of hydrazone building block **21** to which Zn(II) had been previously coordinated (Scheme 2.3).

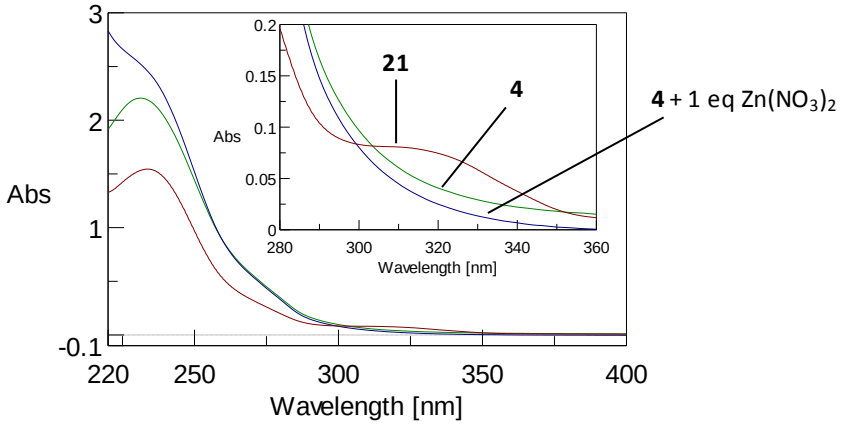
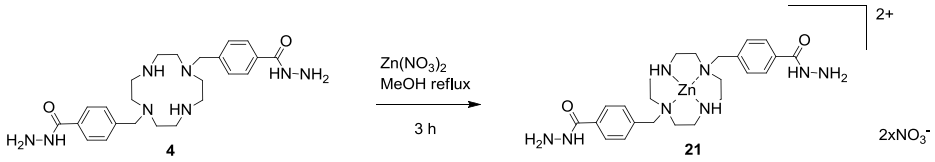


Figure 2.15 UV-vis spectra of **21**, **4** and **4** with 1 equivalent of $Zn(NO_3)_2$. An absorption band at around 320 nm can be observed in the spectrum of pre-synthesized **21** but not when $Zn(NO_3)_2$ is added to **4**. Conditions: 0.2 mM of **4** or **21** in $NH_4^+HCOO^-$ buffer 100 mM pH = 4.0 / acetonitrile (6/4).



Scheme 2.3 Formation of the Zn(II)-hydrazide building block complex **21**.

2.4.2 Molecular recognition by Zn(II)-hydrazide complex

Kimura et *al*. extensively worked with metal complexes and in particular with Zn(II)-cyclen complexes for the recognition of imides such as thymidine.[18] Their work evidenced the coordination of the Zn atom to cyclen through the N atom of the imides which is negatively charged in solution. Hydrogen bonds between the hydrogen atoms connected to the N atoms in the cyclen and the O atoms in the imine also contributed to the recognition process (Figure 2.16). Aromatic pendants on the cyclen structure increased the interactions by means of π-π stacking.[15]

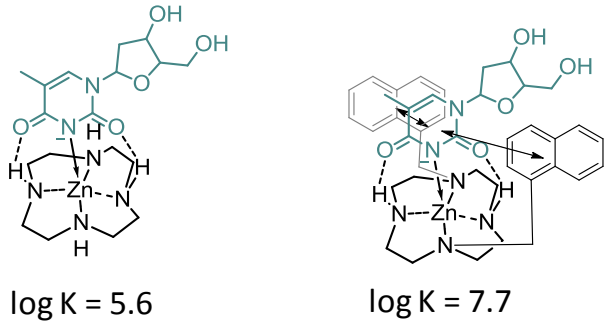


Figure 2.16 Interaction between thymidine and Zn(II)-cyclen complex without and with aromatic pendant arms. A enhanced stability of the complex is achieved by formation of π-π stacking interactions. Conditions: $[ZnL]_{initial}$ = 0.5 mM CH_3CN/EPPS buffer pH = 8.0, 10 mM (1/9) at 25 °C.

These precedents encouraged us to test thymidine for binding to molecule **21**. A ^{1}H-NMR titration of thymidine to **21** showed shifts of the signals from the phenyl and benzyl protons on building block **21** indicating that there was interaction with thymidine (Figure 2.17). These experiments required neutral pH conditions. Acidic condition disrupts the coordination of zinc to cyclen. Indeed, experiments dissolving **21** under the typical acidic conditions used for hydrazone chemistry resulted in the loss of Zn(II) from the building block due to protonation of the N atoms in the cyclen moiety, thereby, weakening the N-Zn(II) coordination.

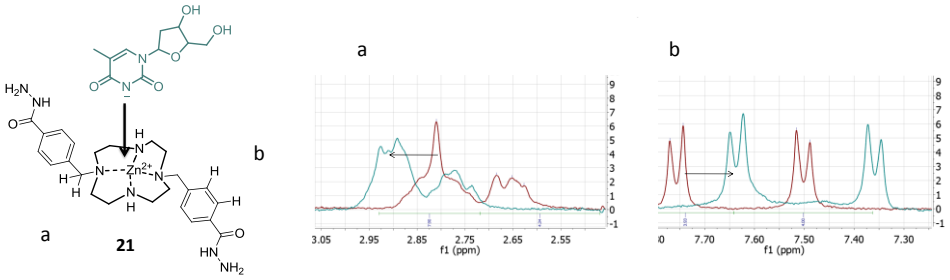


Figure 2.17 Signal shifts in ^{1}H-NMR due to the interaction between thymidine and molecule **21**. Conditions: 12 mM **21**, 12 mM thymidine in HEPES buffer 50mM pH = 7.2. The resonances of protons a and b in the absence and presence of thymidine are shown in red and blue, respectively.

Since binding occurred between **21** and thymidine, we reasoned that also hydrazones containing **21** were likely to interact with this nucleoside, thereby, bringing the possibility

to have a templating effect. Therefore, we set out to perform templating tests on hydrazone libraries made from **21** and dialdehyde molecules at neutral pH.

The compatibility of the aldehydes with building block **21** needed to be checked in order to ensure a proper behavior of the dynamic combinatorial library. For that reason, hydrazone libraries were prepared by mixing 1.0 mM of **21** and 1 equivalent of dialdehyde **5**, **6** or **7** in HEPES buffer (50 mM; pH = 7.2). Regardless of the neutral pH used, no aniline was added on this occasion in order to simplify the library composition. The libraries exhibited precipitation of product, however, only the one with **7** became soluble upon addition of 20% DMSO. The analysis of the library by LC-MS indicated the formation of hydrazones containing Zn(II) (Figure 2.18), alongside the decomposition products associated with the use of **7** (see also Figure 2.7).

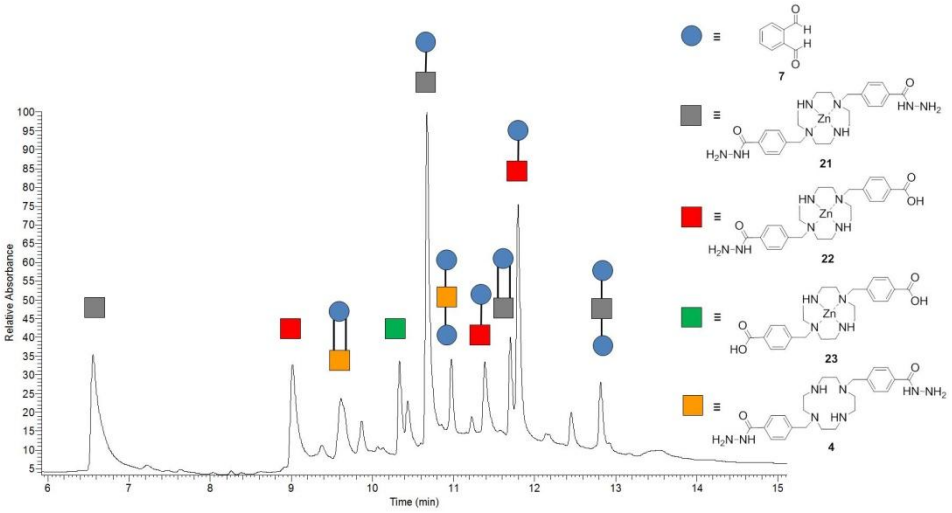


Figure 2.18 Dynamic combinatorial library of Zn-containing structures. Conditions: 1.0 mM of **21**, 1.0 mM of **7** in HEPES buffer 50 mM at pH = 7.2, 20% of DMSO.

This library is the first dynamic combinatorial library of hydrazones prepared using a metal-bound building block.[64] Surprisingly, when thymidine was added to such metal-containing hydrazone libraries, no shift of composition was observed. This suggests a weak and non-selective interactions between thymidine and the library members.

The binding of the powerful metal chelator pyrocatechol violet[65,66] (PV) to **21** was also studied (Figure 2.19). It has been established that Zn(II) can form hexacoordinated complexes.[67] After the tetradentate cyclen ligand has been bound to Zn(II), there is still room for an additional bidentate ligand. Pyrocatechol violet can act as chelating agent for two Zn(II) atoms as it contains two ortho-dihydroxyphenyl groups able to coordinate metals and this fact could enhance the selectivity of PV towards library members containing two metal atoms.

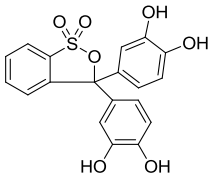


Figure 2.19 Chemical structure of pyrocatechol violet (PV).

A titration of **21** to a solution of PV (0.20 mM) in HEPES buffer (50 mM, pH = 7.2) was performed and the absorbance changes were monitored by UV-vis spectroscopy. The plot of the absorbance change at 450 nm against the concentration of added **21** showed a relatively sharp step (Figure 2.20) indicating a strong binding between PV and **21**. Fitting the data to a theoretical curve for a 1:2 binding model, as other binding experiments suggested,[68,69] afforded a binding constant of $5.7 \cdot 10^5$ M^{-2}.

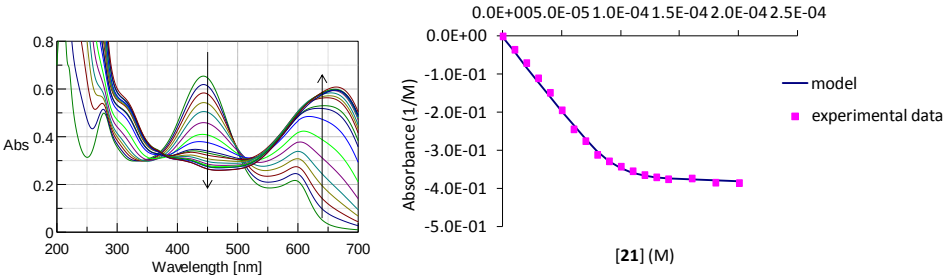


Figure 2.20 UV-vis monitored addition of **21** to pyrocathechol violet (0-2 eq) and fitted absorption values (450 nm). The stoichiometry between pyrocatechol violet and **21** was assumed to be 1:2. The experimental binding constant was found to be $K_{bin} = 5.7 \cdot 10^5$ M^{-2}.

The confirmation of binding of PV to **21** encouraged us to use this dye as a template, expecting it to alter the library distribution. However, when hydrazone libraries were prepared from **21** in combination with **24**, **25** and **26** in presence and absence of PV, no template effect was observed (Figure 2.21).

Although both thymidine and PV proved to be able to coordinate to **21**, none of them afforded any templating response in the hydrazone libraries. These results suggest that, although binding to the library members is probably taking place, the binding site of the host is relatively distant from the rest of the molecule so there is no effective interaction between the guest molecule and the rest of the library member. This lack of interaction between the guest and the host leads to a non-selective interaction between the template and all the library members.

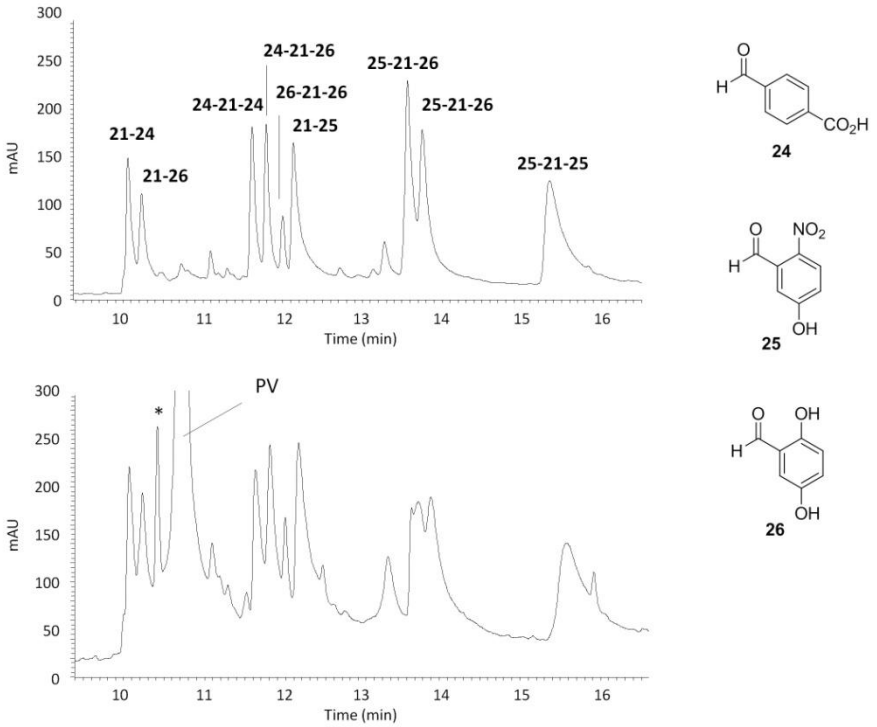


Figure 2.21 Dynamic combinatorial library of linear hydrazones made of **21** (0.25 mM) and monoaldehydes **24-26** (0.2 mM of total aldehyde) in HEPES buffer 50 mM, pH 7.2 (50% v/v CH_3CN) in absence (top) and presence (bottom) of pyrocatechol violet (PV) 0.25mM. The peak indicated by * corresponds to an impurity in PV.

2.4.3 Ester hydrolysis

The absence of template effects makes it difficult to search for catalysts within DCLs and also to ascertain if binding is taking place from the analysis of library distributions. However, it may still be verified whether catalysis occurs by studying the kinetics of the catalyzed reaction. Moreover, any catalysis in this case would probably be performed by all members of the library containing the metal-bound building block. The absence of selectivity of the substrate in binding to the different library members suggests that all of them may have an equal catalytic activity.

Examples of ester hydrolysis catalyzed by Zn(II)-cyclen complexes can be found in the literature.[6,24,25] As reported by Kimura,[20,24] the probable mechanism of catalysis involves initial coordination of a molecule of water to the zinc center, followed by deprotonation to generate a highly nucleophilic $^{-}$OH. The hydroxide anion can then attack the electrophilic carbonyl carbon of the ester molecule to achieve the hydrolysis. Due to the similarity between the building blocks employed in Kimura's work and **21**, it was decided to investigate the potential of our Zn-cyclen complex in the hydrolysis of ester compounds. For that, the hydrolysis of 4-nitrophenyl acetate (4-NPA) in solutions of **4**, **21**, unfunctionalized Zn-cyclen complex (**22**) and zinc nitrate was compared (Figure 2.22). The release of the strongly colored 4-nitrophenolate after hydrolysis of 4-NPA was monitored over time using UV-vis spectrometry and differences in the rate of reaction were observed between the four samples. Neither the solution of compound **4** nor the one of $Zn(NO_3)_2$ accelerated the hydrolysis of 4-NPA. However, the initial rate of hydrolysis of 4-NPA was increased by 4-fold when **21** was present in solution. Surprisingly, Zn(II)-cyclen complex **22** did not increase the hydrolysis rate and it even produced a slightly lower rate of reaction when compared to the blank. This counter intuitive observation could be explained by considering the different Lewis acidities of the metal in both complexes. As previously discussed, the binding constant found by titration for the formation of **21** is much lower than the one of **22**. The difference in K_{bind} values reflects the better electron donation ability of unsubstituted cyclen. This relative electronic richness translates into a less positively charged Zn(II) atom in **22** that therefore has lower Lewis acidity as compared to the Zn(II) atom in **21**. As a result, Zn(II) in **22** cannot deprotonate H_2O as easily as **21**. Moreover, the pK_a of Zn(II)-cyclen-H_2O was found to be around 7.5,[24] and considering that

the experiment was performed at constant pH 7.2, a slight pK_a difference of the metal complex can lead to a big difference in the ratio of the concentration of protonated/deprotonated Zn(II)-cyclen-$^-$OH.

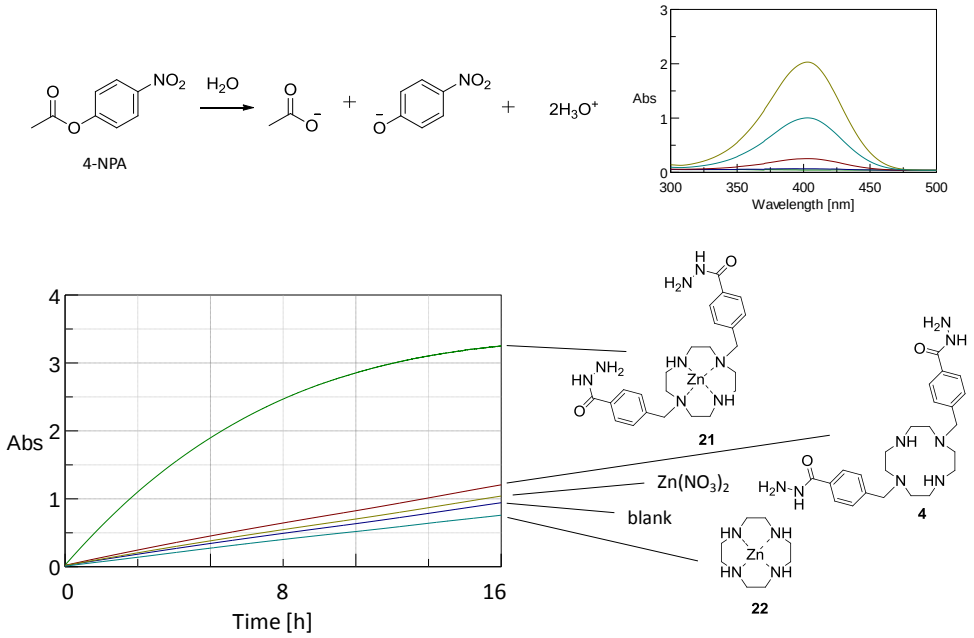


Figure 2.22 Absorption spectra of increasing concentrations of 4-nitrophenolate (top) and kinetics of the reaction of hydrolysis of 4-NPA in presence of **4**, **21**, **22** and $Zn(NO_3)_2$ (bottom). Conditions: 1.25 mM of **4**, **21**, **22** or $Zn(NO_3)_2$ and 0.50 mM of 4-NPA in HEPES buffer 50 mM pH = 7.2, 10% v/v CH_3CN. Absorbance was measured at 400 nm.

The fact that **21** showed catalytic activity, and **22** did not, raised the question whether the hydrazide groups were involved in the mechanism of catalysis, especially since **4** gave slightly higher hydrolysis rate than **22**. To test this hypothesis, we set out to evaluate the catalytic activity of a hydrazone analogue of hydrazide complex **21**. Solubility problems prevented monitoring the hydrolysis of 4-NPA by UV-vis in presence of hydrazones made from **5**, **6** or **7**. The addition of 71% of CH_3CN and 19% v/v of DMSO did not help to dissolve the suspension. Instead, a more soluble aldehyde was used to form a new hydrazone. Molecule **27** (Figure 2.23) was synthesized and 4 eq of it were dissolved together with **21** in HEPES buffer (pH 7.2). The resulting solution containing hydrazone **30** (confirmed by LC-MS) was tested for catalysis.

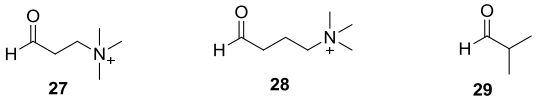


Figure 2.23 Aldehydes used for preparing hydrazone libraries in combination with **21**.

As seen by UV-vis (Figure 2.24) the presence of **30** accelerated the hydrolysis of 4-NPA. After 10 hours the substrate had reached full conversion in the sample containing **30**, while in the blank the concentration of 4-nitrophenolate was still increasing after 28 hours.

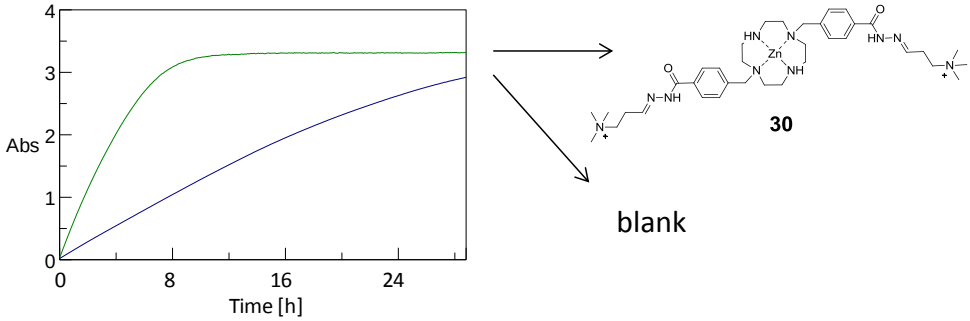


Figure 2.24 Kinetics of the hydrolysis of 4-NPA (0.55 mM) in absence and presence of hydrazone **30** (made from of **21** (1.25 mM) and **27** (5.00 mM)) in HEPES buffer 50 mM, pH 7.2. Monitored by UV-vis spectrometry at 400 nm.

These results imply that the presence of the hydrazide group is not required to achieve catalysis, therefore leaving the door open for obtaining catalytic hydrazone libraries. When a library made by mixing hydrazide **21** and aldehydes **27**-**29** was tested for catalysis, an acceleration of the hydrolysis reaction was also observed. Nevertheless, the increase of the reaction rate in this case was lower than when only aldehyde **27** was added to the reaction mixture (Figure 2.25). The fact that the mixture made from aldehyde **27** performs better than the more diverse hydrazone library suggests that **30** may be a good catalyst. However the LC-MS analysis of this mixture containing **30** revealed the presence of several other compounds (see Experimental, Figure 2.27).

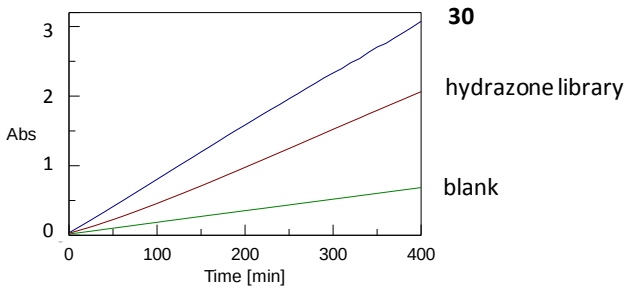


Figure 2.25 Kinetics of the hydrolysis of 4-NPA (0.50 mM) in HEPES buffer 50 mM, pH 7.2, solution of **30** (made from **21** (1.25 mM) and **27** (5.00 mM)) in HEPES buffer and in a hydrazone library made from **21** (0.63 mM), **27** (5.00 mM), **28** (2.5 mM) and **29** (2.5 mM) in HEPES buffer. Monitored by UV-vis spectrometry at 400 nm.

Further work is needed to identify which of these species is responsible for the catalysis. The interaction of 4-NPA with the library can lead, in principle to different situations depending on the relative binding strength of each of the library members to the substrate and their ability to catalyze the transformation into the products. In a situation where the dynamics of the combinatorial library are considered fast with respect to the transformation of the substrate, if the best catalyst is a strong binder, a shift of the library towards formation of more catalyst will happen. If the dynamics of the combinatorial library are too slow, or the best binder is not a good catalyst, the reaction rate of the catalytic reaction will not be greatly increased. Indeed, if a process of transformation of A into B by means of a catalyst C (see Figure 2.26) has a low energy transition state to form AC (TS1) compared to the one to form BC from AC (TS2), the library will have higher chances of being templated since the species AC survives for a relatively long time. On the other hand, if the energy of the transition state TS2 is low compared to TS1, as soon as complex AC is formed it will readily react into BC, rapidly releasing the catalyst and therefore not allowing the library to shift its equilibrium to form more of it. Along with this point, the difference of energy between A+C and AC will also be relevant particularly when TS1<<TS2, as it decides the ratio between bound to A and free C.

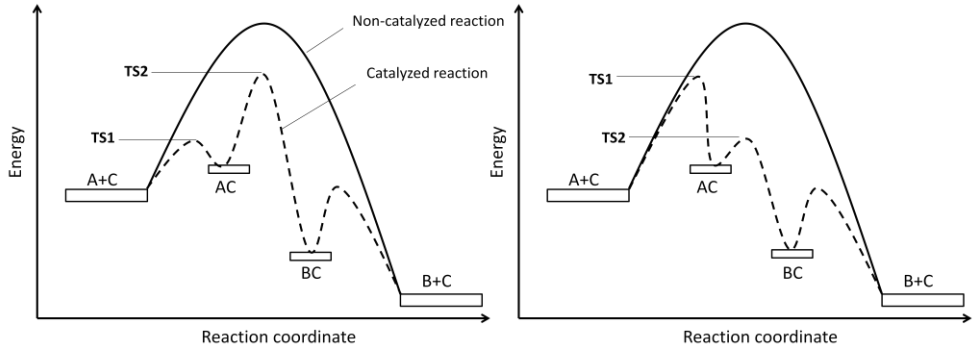


Figure 2.26 Energy profile of catalyzed reactions where binding is fast relatively to catalytic transformation (left) and where binding is slow compared to catalytic transformation (right).

By looking at the data from Figure 2.25, it can be deduced that not all of the hydrazide was converted into **30**. Therefore, either other hydrazones were stabilized by binding to 4-NPA or the dynamics of the library were too slow to allow a shift in the product composition.

2.5 Conclusions

A study of the behavior of hydrazone dynamic combinatorial libraries containing cyclen has been described in this chapter. A decisive role was played by the aldehyde that, depending on its structure, yielded different sizes of cyclic hydrazone structures. The synthesis of a Zn(II)-cyclen hydrazide molecule led to the discovery of an efficient catalytic building block for ester hydrolysis. The formation of the corresponding DCLs was achieved through the use of highly water soluble aldehydes that allowed the corresponding hydrazones to remain in solution. Surprisingly, no templating effects were found in these libraries.

2.6 Experimental

Reagents and solvents

All reagents and solvents were obtained from commercial sources and used without further purification unless specified otherwise.

NMR analysis

NMR spectra were obtained on a Varian AS 400 MHz instrument. ^{1}H chemical shifts are reported as δ in ppm relative to residual protonated solvent resonances. ^{13}C chemical shifts are reported as δ in ppm and measured relative to solvent references. Coupling constants are reported in Hertz.

HPLC analysis

Analytical HPLC was carried out on Hewlett Packard 1050 or 1100 systems coupled with UV detectors and the data were processed using HP Chemstation software. Separations were performed on a reversed phase Waters symmetry C8 column (4.6 x 150 mm, 3.5 µm particle size). Aliquots of 3 µL of library solution were injected. Doubly distilled water, HPLC-S-grade acetonitrile from Biosolve and formic acid were used to prepare the eluents:

eluent A = water + 5.0 % acetonitrile + 0.10 % formic acid

eluent B = acetonitrile + 5.0 % water + 0.10 % formic acid

The eluents for samples containing Zn(II)-hydrazide building block **21** were prepared with trifluoroacetic acid instead of formic acid.

Chromatography was performed at 45 °C using UV detection at 260 nm and a constant flow rate of 1.00 mL / min. The HPLC analysis method was as follows:

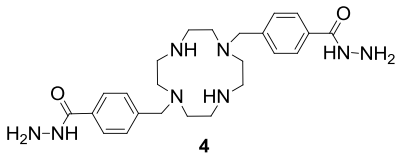
Time (min)	Eluent A (%)	Eluent B (%)
0	100	0
5	89	11
16	33	66
22	0	100
26	0	100
28	100	0
33	100	0

LC-MS analysis

For the LC-MS measurements an Accela High Speed LC system (ThermoFisher Scientific, Courtaboeuf, France) was coupled to a LTQ-Fleet Ion Trap Mass Spectrometer. Mass spectra (positive ion mode) were obtained using the following conditions: sheath gas flow rate 30, aux. gas flow rate 10, sweep gas flow rate 5, ionization spray voltage 3.50 kV, capillary temperature 330 °C, capillary voltage 12 V, tube lens 40 V.

The LC method employed was the same as that one used for HPLC. The flow was split after the LC to allow 0.30 mL / min to enter the mass spectrometer.

Synthetic procedures

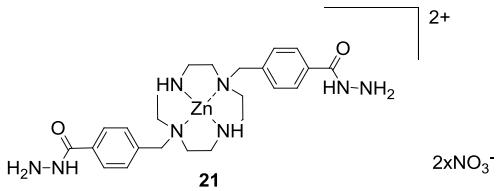


4

4,4'-((1,4,7,10-tetraazacyclododecane-1,7-diyl)bis(methylene))di(benzohydrazide) (**4**)

Glyoxal protected 1,4,7,10-tetraazacyclododecane (190 mg, 1.00 mmol) and methyl-4-(bromomethyl)benzoate (687 mg, 3.00 mmol) were stirred in dry 1.0 mL CH_3CN overnight. After filtering and evaporating the solvent, the crude product was recrystallized from water (500 mg, 77% yield). Hydrazine monohydrate (2.5 mL) was added and the mixture was refluxed for 2 hours. Hot water was added (25 mL) to dissolve the product and the final solution was left at 4 °C to allow the product precipitate (355 mg, 99% yield). M.p. 199.3-201.8 °C. ^{1}H-NMR (400 MHz, DMSO): δ 9.78 (s, 2H), 7.85 (d, *J* = 8.4 Hz, 4H), 7.44 (d, *J* = 8.4 Hz, 4H), 7.19 (br s, 2H), 4.51 (br s, 4H), 3.79 (s, 4H), 2.59 (s, 16H). ^{13}C-NMR (100 MHz, DMSO): δ 166.0 (2xO=C(NH-)-), 142.9 (2xC_{quat}), 132.5 (2xC_{quat}), 129.2 (4xCH), 127.4 (4xCH), 60.9 (2xCH_2), 51.7 (4xCH_2), 47.0 (4xCH_2). HR-MS calcd ($M+H^+$) 469.3034, HR-MS found ($M+H^+$) 469.3029.

4,4'-((1,4,7,10-tetraazacyclododecane-1,7-diyl)bis(methylene))di(benzohydrazide), Zn(II) complex (**21**)

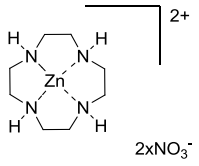


21

Compound **4** (66.4 mg, 0.14 mmol) was dissolved in 5.0 mL MeOH. $Zn(NO_3)_2{\cdot}6H_2O$ (104 mg, 0.35 mmol) was dissolved in 2.0

mL MeOH and added to the solution of **4**. The solution was refluxed for 3 hours, then cooled down to let the product precipitate. The solid was filtered, redissolved in 5 mL MeOH, and the solution was refluxed for 15 minutes. The solution was allowed to cool to room temperature and the solid was filtered and dried under vacuum. The product (89.3 mg, 96% yield) was obtained as a white solid. M.p.: decomposed at 232.2-233.4 °C. ^{1}H-NMR (400 MHz, DMSO): δ 7.90 (d, *J* = 9Hz, 4H), 7.63 (d, *J* = 9Hz, 4H), 4.45 (br s, 2H), 4.19 (s, 4H), 3.36 (m, 4H), 2.29 (m, 8H), 2.86 (m, 4H). ^{13}C-NMR (100 MHz, DMSO/acetone 1/1): δ 161.7 (2xO=C(NH-)-), 145.4 (2xC_{quat}), 133.9 (2xC_{quat}), 131.1 (4xCH), 127.7 (4xCH), 56.3 (2xCH_2), 48.5 (4xCH_2), 42.7 (4xCH_2). HR-MS calcd (M+2x(NO_3^{2-})+H^+) 657.2082, HR-MS found (M+2x(NO_3^{2-})+H^+) 657.2075.

1,4,7,10-tetraazacyclododecane, Zn(II) complex (**22**)



For the synthesis of **22** the same procedure as for **21** was employed but using cyclen instead of **4**. This metal complex has been described in the literature.[70]

M.p. 327.5-328.5 °C. ^{1}H-NMR (400 MHz, DMSO): δ 4.15 (m, 4H), δ 2.74 (m, 8H), δ 2.65 (m, 8H). ^{13}C-NMR (100 MHz, DMSO): δ 48.9 (CH_2).

Preparation of libraries

For a typical hydrazone library, building block **4** (5.00 mM) was dissolved in ammonium formate buffer 100 mM pH 4.0. Aldehyde building block(s) were dissolved in CH_3CN to a total concentration of 10.0 mM. The final sample was prepared by mixing 250 µL of the hydrazide solution and 100 µL of the aldehyde solution. A volume of 350 µL of buffer and 300 µL of CH_3CN were also added to reach the final ratio of buffer/CH_3CN of 6/4. The libraries were stirred at about 800 r.p.m. at room temperature. A similar procedure was used for the libraries made from building block **21**.

LC-MS analysis of the DCL made from 21 and 27

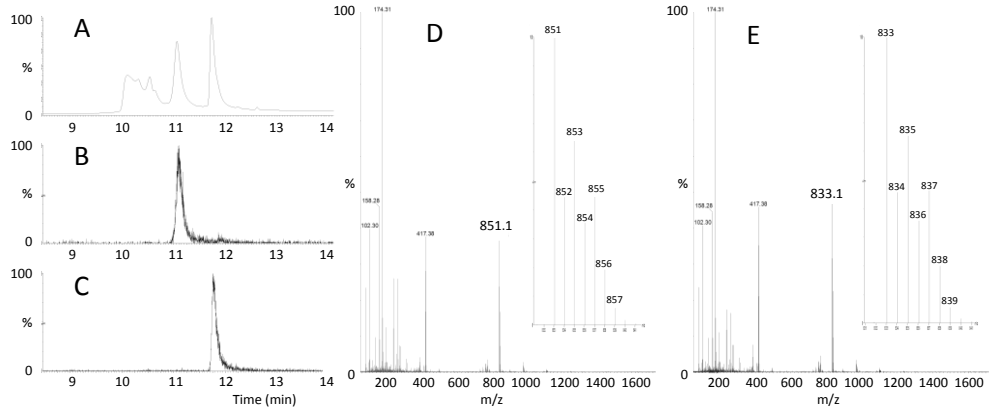


Figure 2.27 UV-vis chromatogram (A) of a solution containing **21** and **27** and the extracted ion chromatograms of m/z = 851 (B) and m/z = 833 (C) corresponding to $C_{36}H_{63}Cl_2N_{10}O_4Zn^+$ (M = 833.370 u.m.a.) and a species with 18 u.m.a. higher mass (probably addition of a molecule of water). Mass spectra of both species are shown (D and E).

Calculation of binding constants

Binding constant between Cu(II) and **4**.

The experimental data points of a plot of A(310nm)/ε against the concentration of Cu(II) were fitted to the theoretical curve

$$A/\varepsilon_l = \frac{(Ccu(II)+Clig+1/Keq)-\sqrt{(Ccu(II)+Clig+1/Keq)\text{^}2-4*Clig*Ccu(II))}}{2} \quad (1)$$

where A = absorbance of the complex, ε_l = molar absorptivity for a set wavelength, $C_{Cu(II)}$ = analytical concentration of Cu(II), C_{lig} = analytical concentration of ligand **4**, K_{eq} = equilibrium constant for the formation of the metal complex.

Equation (1) was obtained as follows:

For the equilibrium reaction **4** + Cu(II) ⇌ **21** $K_{eq} = [\mathbf{21}]/[\mathbf{4}][Cu(II)]$ (2)

A mass balance ensures that $C_{lig} = [\mathbf{4}] + [\mathbf{21}]$ (3) and $C_{Cu(II)} = [Cu(II)] + [\mathbf{21}]$ (4)

Substituting in (3) and (4) in (2), $K_{eq} = [\mathbf{21}]/(C_{lig}- [\mathbf{21}])(C_{Cu(II)}-[\mathbf{21}])$ (5)

Reorganizing (5), $[\mathbf{21}] = K_{eq} (C_{lig}- [\mathbf{21}])(C_{Cu(II)}-[\mathbf{21}]) = (K_{eq}C_{lig}-K_{eq}[\mathbf{21}])(C_{Cu(II)}-[\mathbf{21}])$

$$[\mathbf{21}] = K_{eq} C_{lig} C_{Cu(II)} - K_{eq}[\mathbf{21}] C_{Cu(II)}- K_{eq} C_{lig} [\mathbf{21}] + K_{eq}[\mathbf{21}]\text{^}2 \quad (6)$$

Dividing both by K_{eq}, $[\mathbf{21}]/K_{eq} = C_{lig} C_{Cu(II)} - [\mathbf{21}] C_{Cu(II)}- C_{lig} [\mathbf{21}] + [\mathbf{21}]\text{^}2$ (7)

Taking [**21**] as the unknown, we can arrange (7) in the shape of a quadratic equation,

$$[\mathbf{21}]\text{^}2-(C_{Cu(II)} + C_{lig} + 1/K_{eq})[\mathbf{21}] + C_{lig} C_{Cu(II)} = 0 \quad (8)$$

The Lambert-Beer law, applied to our system, says $A/\varepsilon_l = [\mathbf{21}]$ (9)

Substituting (9) in (8), $(A/\varepsilon_l)^2-(C_{Cu(II)} + C_{lig} + 1/K_{eq})\ A/\varepsilon_l + C_{lig}\ C_{Cu(II)} = 0$ (10)

And solving the quadratic equation (10), we arrive to (1).

$C_{Cu(II)}$ and C_{lig} are known. The values of K_{eq} were guessed until a minimum value of $\sum_n((A/\varepsilon_l)_n^{exp} - (A/\varepsilon_l)_n^{theor})^2$ was found (with n = datapoints for each different addition of Cu(II)). To perform this minimization, Solver tool from Microsoft Office Excel® was used.

A similar equation to (1) was developed for the binding constant between Zn(II) and **4**. Since the titration was monitored by ^{1}H-NMR, the signal observed in this case was δ (ppm) for the benzylic protons in **4**. For this titration, $Zn(NO_3)_2$ (1.20 M) was added to **4** (12.0 mM in 0.70 mL of D_2O).

The binding constant between pyrocatechol violet and Zn(II)-**4** was calculated in the same way. The absorbance was monitored at 450 nm. A solution of $Zn(NO_3)_2$ (5.00 mM) was added to a solution of PV (0.05 mM in HEPES buffer 50 mM, pH = 7.2).

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Chapter 3

Cyclen Derivatives in Dynamic Combinatorial Chemistry. Disulfide Libraries

3.1 Introduction

Reversible disulfide exchange is probably the most popular chemical reaction to form DCLs[1-3] and has been also the most used for the recognition of biological targets.[4-9] It is easy to perform and, importantly, disulfides are chemically compatible with a large number of other functional groups and therefore rarely interfere with molecular recognition. A difference with respect to reversible hydrazone chemistry is that the building blocks used for disulfide exchange have symmetrical linkages (thiols). This means that any two building blocks are able to react with each other, unlike hydrazones that require the union between two different functional groups (hydrazide and aldehyde). As a result, disulfide libraries are often simpler to design than those made from hydrazones. An effective pH suitable for biological applications is an additional reason why disulfide DCLs are widely investigated.

As explained in Chapter 2, 1,4,7,10-tetraazacyclododecane (cyclen) is a cyclic polyamine useful for the binding of metal ions. In this regard, cyclen was chosen to be the core of a dithiol building block with the aim of making disulfide libraries containing cyclen-bound metal ions. The presence of the metal ions in the library members would provide a potential recognition and a catalytic centre. In the literature, no cyclen containing DCLs are known, although other structures containing nitrogen atoms have been used for recognition purposes.[10,11]

In this chapter, the synthesis and application of a cyclen-dithiol building block in disulfide DCLs are described. The addition of metal ions to these libraries is also discussed.

3.2 Study of cyclen-disulfide libraries

3.2.1 Introduction

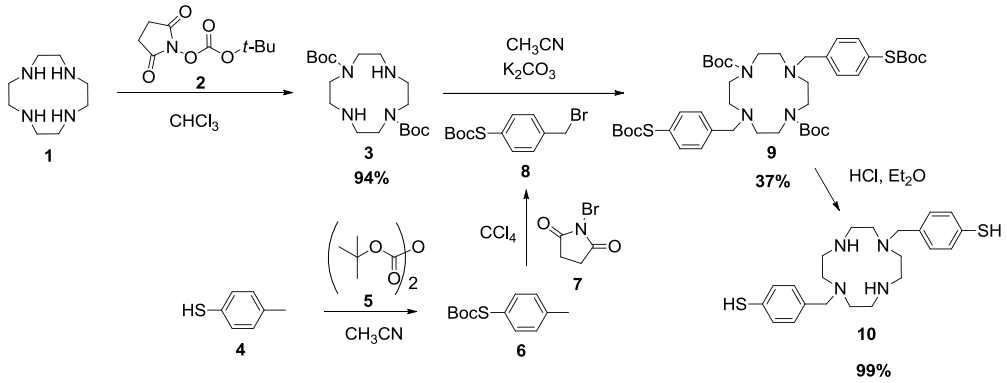
Disulfide libraries containing cyclen were prepared by mixing building blocks equipped with thiol groups. Since thiolates in solution are required for these libraries to be dynamic, a pH between 7 and 9 is normally used. Multiple dithiol building blocks had been tested in our group for the formation of dynamic combinatorial libraries of disulfides,[11-20] so we already accumulated a considerable amount experience in this chemistry. In view of the

promising results previously achieved, a cyclen-bearing building block similar to the one used for hydrazone chemistry (Chapter 2) was synthesized.

3.2.2 Synthesis of a cyclen-thiol building block

A cyclen containing two thiol groups able to form disulfides was designed to be used in disulfide DCLs. Thiol building block **10** was decorated with two phenyl groups that could provide π-π stacking interactions. The aromatic groups increase the possibilities of achieving molecular recognition by offering an apolar environment for organic molecules.

The synthesis of **10** started from bare cyclen **1**. The two more electrophilic nitrogen atoms were protected with *t*-BOC[21] groups. The protected cyclen was reacted with *t*-BOC-protected *p*-(bromomethyl)-thiophenol which had been previously synthesized. Only the unprotected nitrogen atoms were then available to perform the nucleophilic substitution on the brominated carbon atom of reagent **8**. In the last step, an acidic hydrolysis yielded the final product with an overall yield of 35% (Scheme 3.1).



Scheme 3.1 Synthetic route of dithiol building block **10**.

3.2.3 Cyclen containing disulfide libraries

Thiol libraries of 1.00 mM building block concentration were prepared by dissolving **10** in borate buffer at pH 8.0. After 3 days, 80% of **10** had been oxidized into disulfides. The disulfide composition at this time consisted of 95% of cyclic dimer and a 5% of cyclic monomer **11** (Figure 3.2). The presence of the latter species was unexpected. Due to the high degree of strain in its structure, it was considered to be a high-energy species.

Indeed, this disulfide was not a thermodynamically stable species but the result of a kinetically controlled reaction. This fact was evident from an analysis of a similarly prepared library in presence of Cu(II) as an oxidation catalyst which revealed an increased proportion of cyclic monomer compared to the library in absence of the metal (Figure 2.2).

As a strategy to increase the diversity of the dynamic disulfide combinatorial network, other dithiol molecules were also included to participate in the formation of mixed disulfide species. Building blocks **13** and **14** were mixed together with **10** to form a ternary library of disulfides. An assortment of cyclic dimeric, trimeric and tetrameric structures was identified in the mixture (Figure 3.3). This diversity of products is a desirable feature in a library and prompted an initial screening of binding to potential guest molecules.

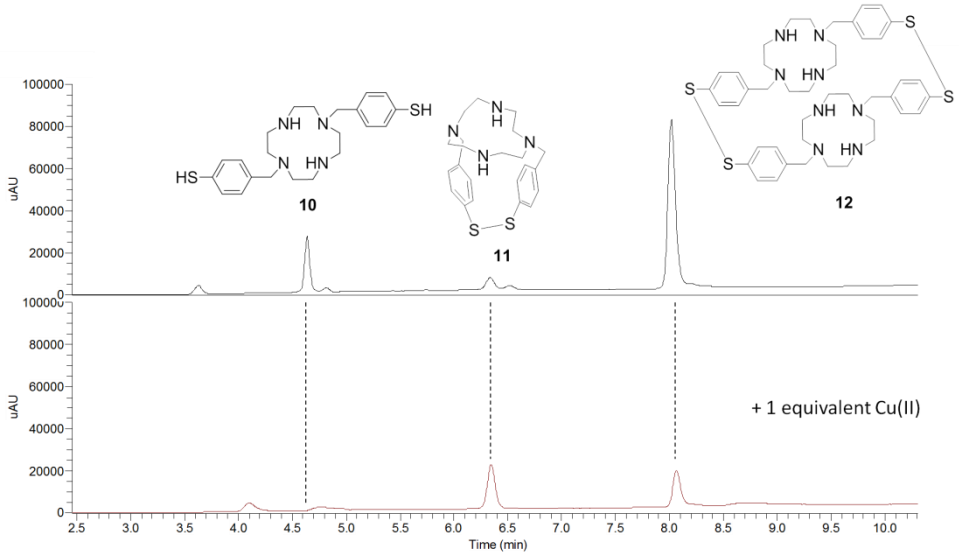


Figure 3.2 HPLC analysis of a disulfide library made from building block **10** in presence (above) and absence (below) of Cu(II) as a catalyst of thiol oxidation. A broad peak probably corresponding to the presence of larger oligomers was observed during washing of the column after analysis of the Cu(II) containing DCL. Conditions: 1.00 mM of **10** in borate buffer 50 mM, pH = 8.0.

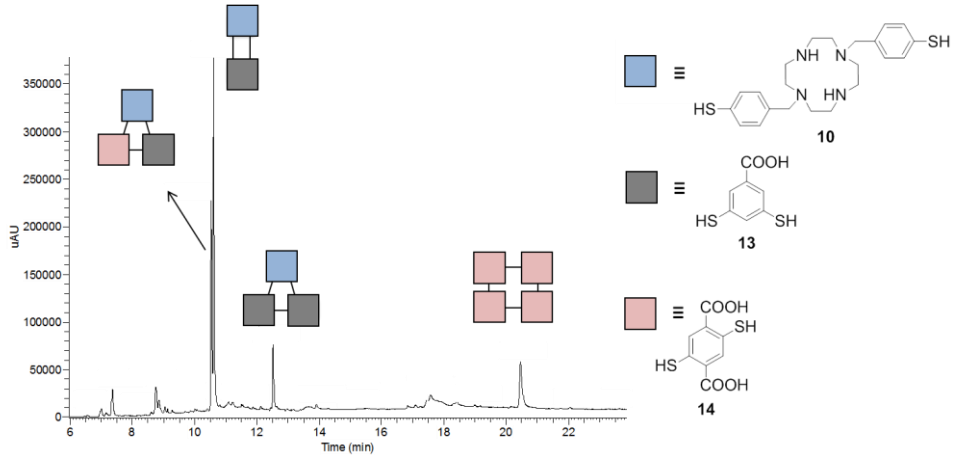


Figure 3.3 HPLC analysis of a disulfide library made from building blocks **10**, **13** and **14** after 8 days. Conditions: 1.00 mM of total building block concentration in borate buffer 50 mM, pH 8.0.

With the aim of shifting the library composition, some molecules were tested as templates (Figure 3.4). While **15** was known to amplify the cyclic trimer made from **13**, **16** had been reported to bind to other carboxylic-containing disulfide library members. Molecules **1**, **15**-**18** are positively charged in aqueous media and / or contain aromatic residues that are able to provide electrostatic and π-π stacking interactions with the library members. However, none of those molecules was able to produce any template effect in the library made from **10**, **13** and **14**.

Although no response from the library was detected in presence of any of the added molecules, the ability of **10** to combine with other thiol molecules and provide disulfide structures, encouraged us to continue with the study of these libraries for the formation of catalysts. For this purpose, metal ions were tested in combination with disulfide libraries.

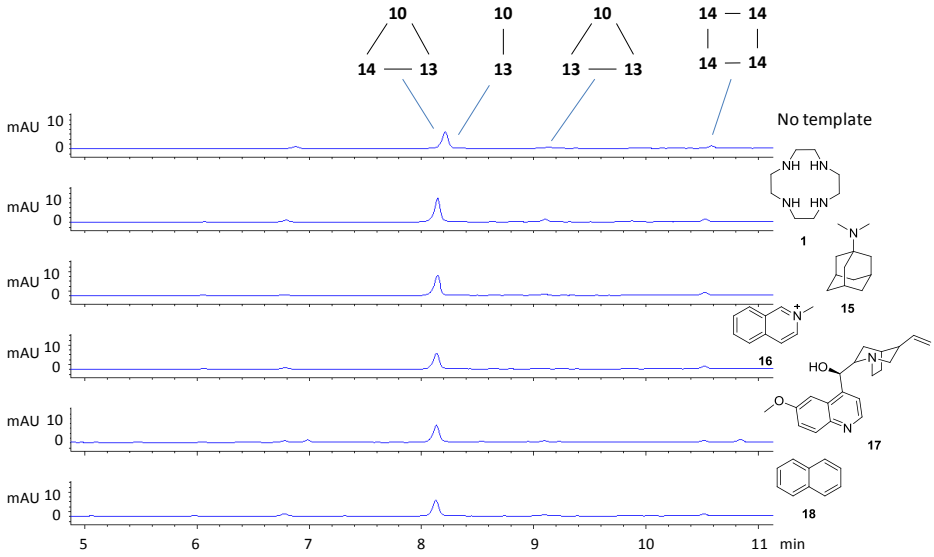


Figure 3.4 The HPLC analysis of libraries made from **10**, **13** and **14** in presence of **1**, **15**-**18** did not reveal any templating effect. The main peak corresponds to the cyclic dimer made from **10** and **13**. Conditions: 0.33 mM concentration of each building block and 1.00 mM concentration of template in borate buffer 50 mM, pH 8.0. The difference in the appearance of the top trace and that shown in Figure 2.3 is due to the fact that they were obtained using different LC instruments.

3.3 Metal salts and disulfides

The presence of the cyclen moiety within **10** gives the possibility to bind metal ions to the dithiol building block. Such metals could act as recognition centers for guest molecules and, in some cases, also as catalysts. Up to now, no examples of aqueous disulfide libraries in the presence of transition metals have been reported, although some examples are found in organic solvents.[22-24] Interactions between sulfur and metal ions[25-27] are known to occur and could lead to the inactivation of the metal[28] and interference with the disulfide exchange process. Our aim was to explore the compatibility of metal salts to be used within the disulfide libraries without disturbing their dynamic nature.

Initial tests combining metal ions with **10** were performed to obtain some information about the kinetics of the ligand coordination to the metals. Solutions of **10** in borate buffer were prepared and Co(III), Ni(II), Fe(III), Cu(II) or Zn(II) salts were added to them. The UV-vis absorption spectra of the solutions were recorded over time revealing that the coordination was slow, similarly to the coordination of metals to the hydrazide building

block bearing cyclen (Figure 3.5). However, the HPLC traces of disulfide libraries made from **10** and metal ions, showed some effect of the metals on the product distribution of the libraries (Figure 3.6). Cu(II) increased the amount of cyclic monomer as previously stated, as a result of a catalytic thiol oxidation process, but the total peak area observed in the chromatogram consistently decreased as compared to the sample in absence of metal. Presumably, the fast oxidation of thiols led to the formation of larger oligomers which eluted during the column washing step. Ni(II) and Co(II) behaved similarly, slightly accelerating the oxidation process. Only Ga(III) seemed to delay the thiol oxidation process. In any case, the metal ions by themselves did not produce any templating effect in the library.

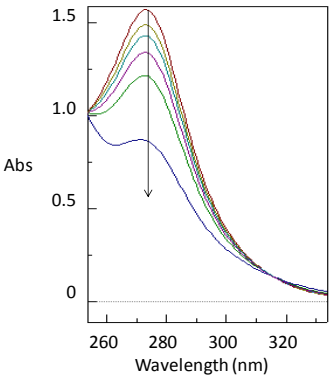


Figure 3.5 The binding of Cu(II) to **10** was monitored over time by UV-vis spectrometry. The shown spectra correspond to time measurements of 5, 10, 20, 35, 60 and 150 minutes. Conditions: 0.10 mM **10** and 0.02 mM $Cu(NO_3)_2$ in borate buffer 50 mM pH 8.0.

Attempts to achieve metal-mediated templating effects were carried out. The successful thymidine recognition by the hydrazide building block prompted us to test this molecule in disulfide DCLs. For that purpose, 1 eq of $Zn(NO_3)_2$ was added to a solution of **10** in borate buffer (in D_2O) 50 mM, pD 9.0. At this stage, a slight turbidity appeared probably coming from the formation of insoluble $Zn(OH)_2$. The addition of thymidine to that suspension produced more precipitation. This phenomenon was impossible to revert by addition of organic solvents, and therefore the experiment was abandoned at that point.

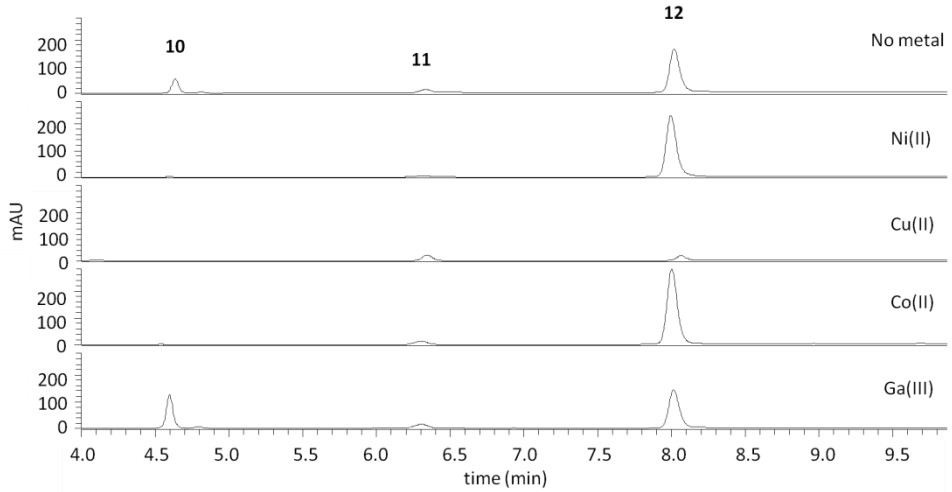


Figure 3.6 The presence of metal ions (1 eq) in the disulfide libraries made of **10** (1.00 mM) in borate buffer 50 mM pH 8.0 did not affect the distribution of the library members. Cu(II) accelerated the oxidation process as well as Ni(II) and Co(II) although the latter to a lesser extent. Ga(III) slowed down the formation of disulfides. Libraries were analyzed after 48h.

These negative results forced us to change our strategy once again and make use of a pre-synthesized metal complex to prepare disulfide libraries. After the successful complexation of Zn(II) to a hydrazide building block, the formation of the analogous dithiol-Zn(II) complex was attempted. Following a similar procedure to the synthesis of the Zn-hydrazide complex from Chapter 2, building block **10** and an excess of $Zn(NO_3)_2$ were mixed in boiling deoxygenated MeOH to obtain building block **19** (Figure 3.7).

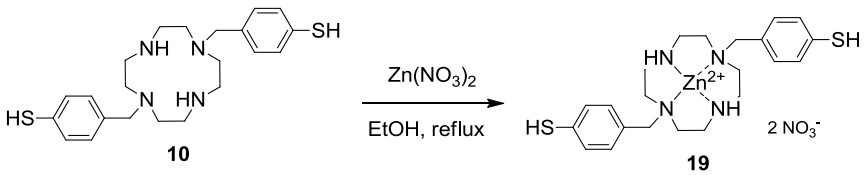


Figure 3.7 Synthesis of Zn-dithiol building block **19**.

The preparation of disulfide libraries from the metal complex **19** consistently resulted in precipitation upon oxidation of the thiol groups. The addition of isopropanol, EtOH or MeOH to the solution failed to re-dissolve the precipitate. Other libraries containing **19** in

combination with different and more soluble thiol building blocks were prepared with the hope of forming soluble dithiols. Cysteine, dithiols **13** and **14** or ethanethiol were tested with no positive results. Libraries with a lower concentration (0.2 mM) of **19** were also tested but precipitation was still observed.

Unfortunately, these persistent solubility problems led us to abandon the development of disulfide libraries using Zn-dithiol building block **19**. A possible solution for this precipitation issue might involve the use of charged building blocks with better solubilization properties. Alternatively, the modification of building block **19** by addition of polar functional groups could also solve the problem. On the other hand, the variation of the metal without further modification of the building blocks is not expected to change the solubility characteristics of the library, unless additional coordination by soluble ligands is occurring.

3.4 Conclusions

A study of the behavior of disulfide dynamic combinatorial libraries containing cyclen has been presented in this chapter. Dithiol molecules containing cyclen were mixed with other thiol building blocks which, upon oxidation, formed disulfide dynamic combinatorial libraries containing cyclic dimers, trimers and tetramers. The use of metal salts in these libraries was restricted to previously synthesized metal-dithiol complexes in view of the slow kinetics for the formation of the complex. The use of Zn-dithiol building block **19** for the formation of metal containing disulfide combinatorial libraries was frustrated by precipitation problems.

3.5 Experimental

Reagents and solvents

All reagents and solvents were obtained from commercial sources and used without further purification unless otherwise specified.

NMR analysis

NMR spectra were obtained on a Varian AS 400 MHz instrument. ^{1}H chemical shifts are reported as δ in ppm relative to residual protonated solvent resonances. ^{13}C chemical shifts are reported as δ in ppm and measured relative to solvent references. Coupling constants are reported in Hertz.

HPLC analysis

Analytic HPLC was carried out on Hewlett Packard 1050 or 1100 systems coupled to UV detectors and the data were processed using HP Chemstation software. Separations were performed on a reversed phase Waters Symmetry C8 column (4.6 x 150 mm, 3.5 µm particle size). Aliquots of 3 µL of library solution were injected. Doubly distilled water, HPLC-S-grade acetonitrile from Biosolve and formic acid were used to prepare the eluents:
eluent A = water + 5.0 % acetonitrile + 0.10 % formic acid
eluent B = acetonitrile + 5.0 % water + 0.10 % formic acid
Chromatography was performed at 45 °C using UV detection at 260 nm and a constant flow rate of 1.00 mL / min. The HPLC analysis method was as follows:

Time (min)	Eluent A (%)	Eluent B (%)
0	100	0
5	89	11
16	33	66
22	0	100
26	0	100
28	100	0
33	100	0

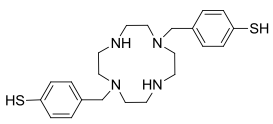
LC-MS analysis

For the LC-MS measurements an Accela High Speed LC system (ThermoFisher Scientific, Courtaboeuf, France) was coupled to a LTQ-Fleet Ion Trap Mass Spectrometer. Mass spectra (positive ion mode) were obtained using the following conditions: sheath gas flow rate 30, aux. gas flow rate 10, sweep gas flow rate 5, ionization spray voltage 3.50 kV, capillary temperature 330 °C, capillary voltage 12 V, tube lens 40 V.

The LC method employed was the same as that one used for HPLC. The flow was split after the LC to allow 0.30 mL / min to enter the mass spectrometer.

Synthetic procedures

4,4'-((1,4,7,10-tetraazacyclododecane-1,7-diyl)bis(methylene))dibenzenethiol (**10**)



1. Boc protection of cyclen (**1**):[21] To a solution of 1,4,7,10-tetraazocyclododecane (cyclen) (1.43 g, 8.00 mmol) in 60 mL of chloroform, N-(*tert*-butoxycarbonyloxy) succinimide (3.44 g, 16.0 mmol) was added. The reaction mixture was stirred for 2 days at r.t. The solvent was then removed by under vacuum and 60 mL NaOH (3.0 M) was added to the remaining residue. An extraction with 3 x 30 mL chloroform was performed, the organic extracts were combined, dried over $MgSO_4$ and the solvent was removed under vacuum. 1,7-di-Boc protected cyclen was obtained as a white solid (2.80 g, 94%). ^{1}H-NMR ($CDCl_3$, 200 MHz): 3.30 (m, 10H), 2.69 (m, 8H), 1.37 (s, 18H).

2. Boc protection of methylthiophenol (**4**):[29] A solution of Boc-anhydride (2.16 g, 14.4 mmol) in 50 mL of acetonitrile was cooled in an ice bath. A solution containing DMAP (0.59 g, 4.80 mmol) and TEA (2.94 g, 30.0 mmol) in 40 mL of acetonitrile was added to the solution of Boc-anhydride. *p*-Methylthiophenol (1.20 g, 9.60 mmol) was added gradually and the reaction mixture was stirred overnight at r.t. The solvents were then removed under vacuum. The remaining yellow solid was dissolved in 20 mL of chloroform and washed with 3 x 30 mL water and the organic phase dried over $MgSO_4$. The solvent was removed under vacuum and the residue was purified by column chromatography using

Hex:AcOEt (40:1) as eluent to obtain 0.80 g (50% yield) of O-*tert*-butyl S-*p*-tolyl carbonothioate. ^{1}H-NMR ($CDCl_3$, 400 MHz): 7.40 (d, *J* = 8.0Hz, 2H), 7.19 (d, *J* = 8.0Hz, 2H), 2.36 (s, 3H), 1.50 (s, 9H). ^{13}C-NMR($CDCl_3$, 100 MHz): 168.2 (C_{quat}), 139.6 (C_{quat}), 134.9 (2xCH), 129.9 (C_{quat}), 125.0 (2xCH_2), 85.4 (C_{quat}), 28.2 (3xCH_3), 21.3 (CH_3).

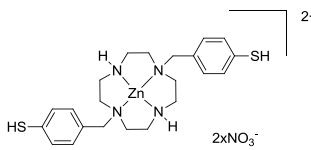
3. Bromination of O-*tert*-butyl S-*p*-tolyl carbonothioate (**6**): N-bromosuccinimide (1.27 g, 7.13 mmol) was added to 20 mL of carbon tetrachloride. O-*tert*-butyl S-*p*-tolyl carbonothioate (1.50 g, 6.70 mmol) and benzoyl peroxide (58 mg, 0.24 mmol) were then added and the reaction mixture was refluxed for 2 hours. After the solution cooled down, the remaining solid was filtered and the solvents were evaporated in vacuo. The crude product was purified by column chromatography using Hex:AcOEt (20:1) as eluent to obtain S-(4-(bromomethyl)phenyl) O-*tert*-butyl carbonothioate (0.76 g, 59%) as a white solid. M.p. 85.3 – 86.2 °C. ^{1}H-NMR ($CDCl_3$, 400 MHz): 7.50 (d, *J* = 8.0Hz, 2H), 7.41 (d, *J* = 8.0Hz, 2H), 4.47 (s, 2H), 1.52 (s, 9H). ^{13}C-NMR($CDCl_3$, 100 MHz): 167.4 (C_{quat}), 138.9 (C_{quat}), 135.0 (2xCH), 129.7 (2xCH), 127.1 (C_{quat}), 85.8 (C_{quat}), 32.5 (CH_2), 28.2 (3xCH_3). HR-MS calcd (M-Na^+) 324.9868, found 324.9855.

4. Synthesis of di-*tert*-butyl 4,10-bis(4-((*tert*-butoxycarbonyl)thio)benzyl)-1,4,7,10-tetraazacyclododecane-1,7-dicarboxylate (**9**): In a round bottomed flask, S-(4-(bromomethyl)phenyl) O-*tert*-butyl carbonothioate (0.82 g, 2.71 mmol), 1,7-(bis(*tert*-butoxycarbonyl)-1,4,7,10-tetraazacyclododecane (0.31 g, 0.83 mmol) and Na_2CO_3 (0.90 g, 8.50 mmol) were added to 15 mL of DMF. The mixture was stirred overnight at r.t. It was then filtered, diluted with 100 mL brine and extracted with 3 x 100 mL dichloromethane. The organic phase was washed with 2 x 100 mL brine, dried over $MgSO_4$ and the solvents were removed in vacuo. The final residue was purified by column chromatography on alumina with Hex:AcOEt (20:1) and (1:1) to obtain 0.33 g (49%) of product. ^{1}H-NMR ($CDCl_3$, 300 MHz): 7.44 (d, *J* = 7.5Hz, 4H), 7.31 (d, *J* = 7.5Hz, 4H), 3.62 (s, 4H), 3.40 (m, 8H), 2.67 (m, 8H), 1.50 (s, 18H), 1.30 (s, 18H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 176.7 (2xC_{quat}), 167.5

($2xC_{quat}$), 143.0 ($2xC_{quat}$), 134.9 (4xCH), 129.6 (4xCH), 127.2 ($2xC_{quat}$), 85.6 ($2xC_{quat}$), 85.3 ($2xC_{quat}$), 64.1 ($2xCH_2$), 41.8 ($2xCH_2$), 28.5 ($6xCH_3$), 28.1 ($6xCH_3$). HR-MS calcd ($M\text{-}H^+$) 817.4238, found 817.4235.

5. Deprotection of di-*tert*-butyl 4,10-bis(4-(((*tert*-butoxycarbonyl) thio)benzyl)-1,4,7,10 -tetraazacyclododecane-1,7-dicarboxylate (**9**): (80 mg, 0.10 mmol) of **9** were dissolved in 10 mL of degassed ethanol and the solution was cooled in an ice bath under a N_2 atmosphere. HCl conc. (10 mL) was added dropwise through a septum and the mixture was stirred overnight. Solvents were removed in vacuo and the white solid residue was washed with degassed ethanol yielding 41 mg, (99%) of 4,4'-((1,4,7,10-tetraazacyclododecane-1,7-diyl)bis(methylene))dibenzenethiol. M.p. 227.0 -230.2 °C. ^{1}H-NMR (D_2O, 400 MHz): 7.42 (d, *J* = 8.1Hz, 4H), 7.25 (d, *J* = 8.1Hz, 4H), 3.72 (s, 4H), 3.13 (m, 8H), 2.88 (m, 8H). ^{13}C-NMR($(CD_3)_2NC(O)D/D_2O$, 50 MHz): 133.6 ($2xC_{quat}$), 133.4 ($2xC_{quat}$), 132.9 (4xCH), 130.3 (4xCH), 57.6 ($2xCH_2$), 48.9 ($4xCH_2$), 44.4 ($4xCH_2$). HR-MS calcd ($M\text{-}H^+$) 415.1985, found 415.1978.

4,4'-((1,4,7,10-tetraazacyclododecane-1,7-diyl)bis(methylene))dibenzenethiol, Zn(II) complex (**19**)



For the synthesis of this compound the same procedure as described for molecule **21** in Chapter 2 was employed. M.p. 293.3 - 294.7 °C. ^{1}H-NMR (200 MHz, D_2O): δ 7.30 (d, *J* = 8.0 Hz, 4H), 7.09 (d, *J* = 8.0 Hz, 4H), 3.59 (s, 4H), 3.07 (m, 10H), 2.79 (m, 8H). ^{13}C-NMR (100 MHz, DMSO): δ 166.0 (2xO=C(NH-)-), 142.9 ($2xC_{quat}$), 132.5 ($2xC_{quat}$), 129.2 (4xCH), 127.4 (4xCH), 60.9 (4xCH), 51.7 (4xCH), 47.0 (4xCH). HR-MS calcd ($M\text{-}H^+$) 479.1266, found 479.1276.

Dithiol building blocks **13**[12] and **14**[15] were prepared as described previously.

Preparation of libraries

For a typical disulfide library, building block **1** (1.00 mM) was dissolved in borate buffer 50 mM pH 8.0. The final sample was prepared by mixing 800 µL of the disulfide solution and 200 µL of IPA. The solutions containing the libraries were stirred at about 800 r.p.m. at room temperature.

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Chapter 4

Transient Substrate-Induced Catalyst Formation in a Dynamic Molecular Network

This Chapter has been published:

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4.1 Introduction

Most of the chemical reactions in Nature are mediated by enzymes. The concentrations of these enzymes are controlled in space and time through elaborate regulatory mechanisms, giving rise to complex functional behavior that is essential to biology.[1] Synthetic catalysts have been developed, many of which complement enzymatic catalysis with respect to types of reactions and substrate scope. Despite recent progress in supramolecular,[2-9] allosteric[10-12] and switchable[13-15] catalysts, temporary control over catalyst concentration remains underdeveloped in synthetic systems, imposing limits on the functional potential of catalysis. We reasoned that more elaborate control over synthetic catalysts may be achieved using dynamic molecular networks.[16] Such networks have been mostly used for creating dynamic combinatorial libraries (DCLs),[17-19] which are powerful tools for discovering synthetic receptors,[20] ligands for biomolecules,[21-23] self-assembling materials,[24-26] interlocked molecules[27] and sensors,[28] as has already been described in Section 1.1. However, dynamic combinatorial approaches to catalysis are relatively underexplored.[29-32] In this chapter, a catalytic dynamic molecular network based on reversible disulfide chemistry is presented. The catalytic molecular network has an important characteristic when compared to the traditional catalytic systems: the catalyst concentration is controlled by the substrate of the chemical reaction on which the catalyst operates. This chapter describes how disulfide libraries are screened for templating effects using a reaction substrate and a transition-state analog. The effect of the substrate on the distribution of the dynamic combinatorial library and the effect of the DCL on the substrate transformation is then studied. Finally, in order to shed light on the mechanism of the catalyzed reaction, the order in substrate and catalyst is determined.

4.2 Developing the strategy

A reaction substrate may act as a template for the production of its own catalyst within a dynamic molecular network by binding to this catalyst, thereby shifting the equilibrium of the network towards the catalyst formation (Figure 4.1). However, catalytic activity requires that the catalyst binds to and stabilizes the transition state of the reaction more than it stabilizes the substrate. Hence, our approach to substrate-triggered catalyst

formation relies on screening a series of DCLs for their ability to produce molecules that bind both the substrate and the transition state of a chemical reaction. The potential for substrate and transition-state binding may be assessed in two separate screening steps. While substrate affinity may be evaluated directly by searching for library members which concentration increases upon exposure to this substrate, the transient character of the transition state of a chemical reaction makes an analogous direct assessment of transition-state binding impossible. However, by using a stable molecule that resembles the structural and electronic nature of the transition state (a transition-state analog or TSA), an indirect assessment of transition-state binding may still be obtained (Figure 4.2).

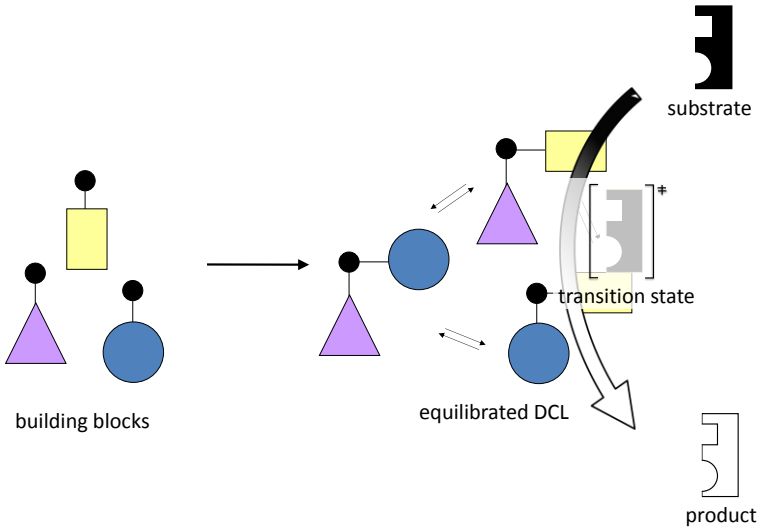


Figure 4.1 Graphic representation of a dynamic combinatorial library in which catalysis takes place.

Following the two screening steps, systems may be identified for which the substrate and the transition-state analog amplify the same species. Such systems may show two different outcomes that are in principle equally likely to happen: the prospective catalyst binds the transition state stronger than the substrate and catalysis is achieved, or vice versa, so there is no catalytic activity.

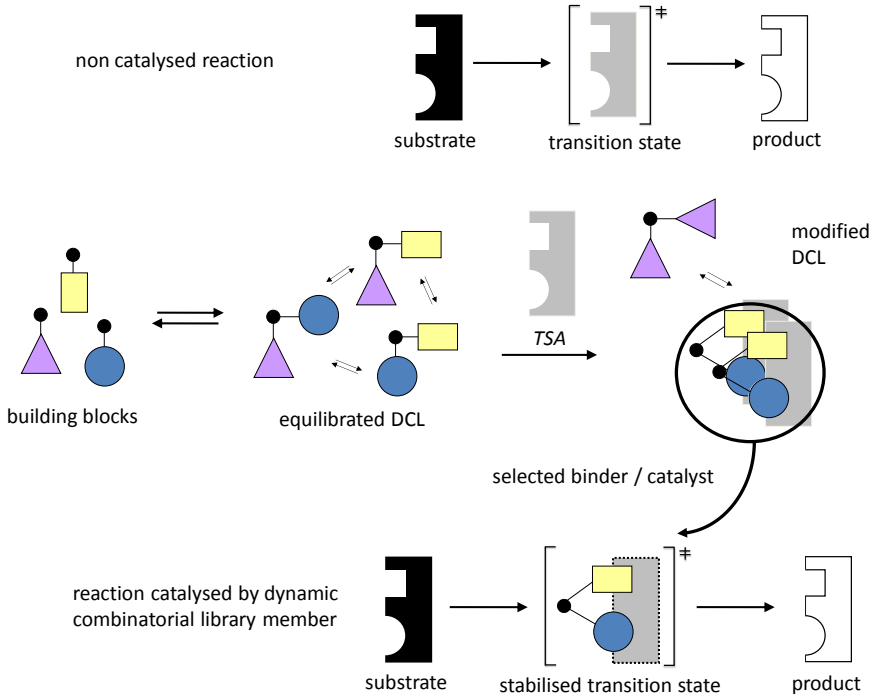
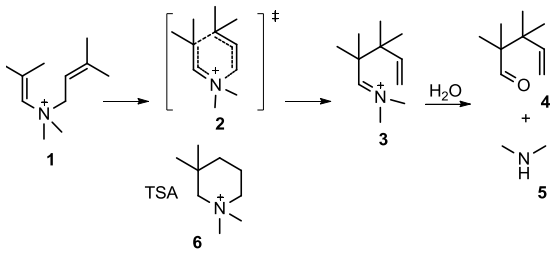


Figure 4.2 General scheme showing the strategy employed to discover a catalyst in a dynamic combinatorial library using a transition-state analog (TSA) to select transition state binders from the library members.

4.3 Templating effects of substrate and transition-state analog

We set out to screen for molecular networks that are capable of catalyzing the intramolecular aza-Cope rearrangement of **1** in aqueous solution[34,35] (Scheme 4.1). As previously described in Section 1.3, the aza-Cope rearrangement of **1** was found to proceed faster when an apolar pocket was available in the aqueous solution. The concerted reaction proceeds through a six-membered cyclic transition state (**2**) giving an unstable enammonium ion product (**3**) that reacts rapidly with water to give aldehyde **4** and dimethylamine (**5**).[34,35] We used cyclic ammonium salt **6** as the transition-state analog for the rate-determining aza-Cope rearrangement step.

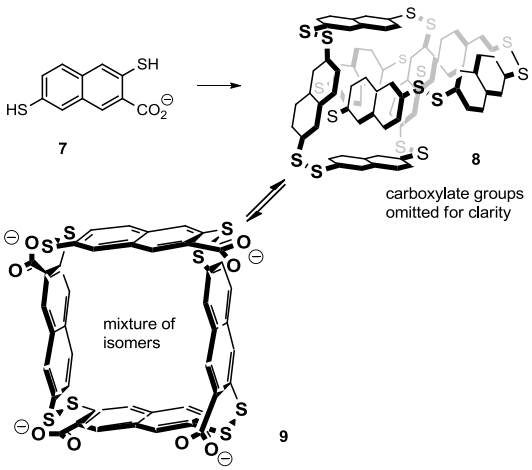


Scheme 4.1 Catalyzed aza-Cope rearrangement and its transition-state analog.

In a previous work, our group developed aqueous DCLs made from dithiol building blocks which were able to respond to the presence of quaternary ammonium salts.[16] With these results in mind, we decided to screen several DCLs based on disulfide chemistry for binding to substrate **1** and transition-state analog **6**. For that purpose, solutions containing different dithiol building blocks in borate buffer (50 mM, pH 8.0) were prepared, and the product distribution of the libraries was compared in absence and presence of **6** and **1**.

Disulfide DCLs can be generated by oxidizing thiol building blocks using oxygen from the air. Disulfide exchange is mediated by nucleophilic attack of disulfides by thiolate anions; hence, the DCLs remain dynamic as long as there is thiolate anion present in solution.[36] In order to guarantee the dynamic nature of the molecular networks, the experiments were performed at a constant redox state, where about two-thirds of the thiol groups had been oxidized to disulfides. To maintain a fixed redox state, sodium perborate was used to partially oxidize the thiols and then the samples containing the disulfide libraries were kept in an oxygen-free atmosphere (see experimental part). Promising results were obtained for a small DCL prepared by partially oxidizing an aqueous solution of building block **7** (Scheme 4.2).

In the absence of any template molecules, the dynamic mixture was dominated by a set of isomeric 2-catenanes (**8**) consisting of two interlocked tetrameric macrocycles, as reported previously (Figure 4.3, top).[36] Only minor amounts of four isomeric tetramer macrocycles (**9**) were obtained.



Scheme 4.2 Oxidation of dithiol building block **7** gives rise to a small dynamic molecular network containing catenanes **8** and macrocycles **9** as mixtures of regioisomers.

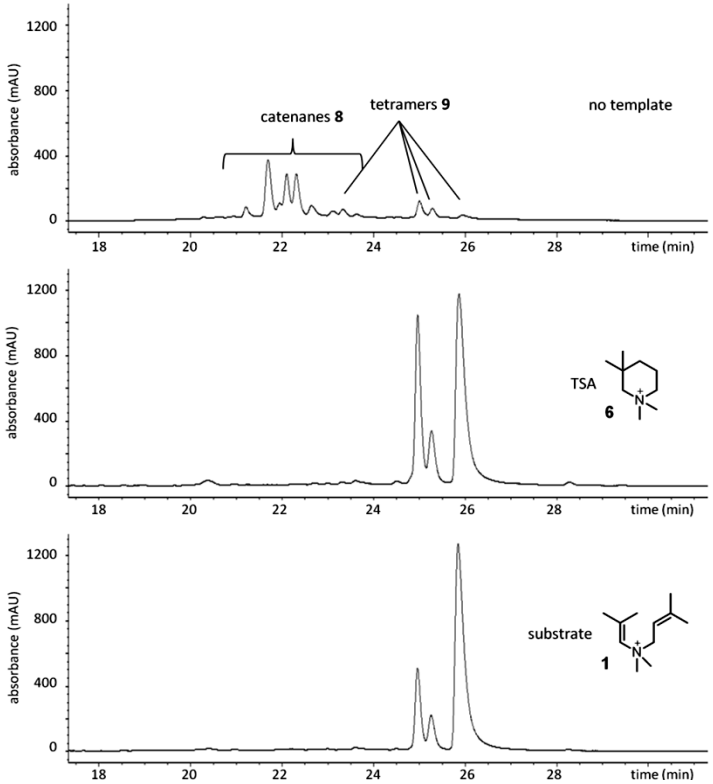


Figure 4.3 Templating results. HPLC analysis of a small dynamic combinatorial library made from **7** (8.00 mM in 50 mM borate buffer, pH 8.0) in the absence of template (top); in the presence of 2.00 mM **6** (middle) and in the presence of 2.00 mM **1** (bottom).

Exposing the dynamic molecular network to transition-state analog **6** induced a dramatic shift in the product distribution, giving full conversion to the tetrameric macrocycles (Figure 4.3, middle). Exposing the network to substrate **1** gave an almost identical result (Figure 4.3, bottom), suggesting that the tetramers are capable of binding both substrate and transition state of the aza-Cope rearrangement, a necessary condition to obtain catalysis.

4.4 Effect of the dynamic combinatorial library on the aza-Cope rearrangement

We proceeded then to study the effect of the dynamic molecular network on the kinetics of the aza-Cope rearrangement. Monitoring the appearance of the signals of the dimethylamine product by ^{1}H-NMR (Figure 4.4) revealed that the aza-Cope rearrangement in the presence of the dynamic disulfide mixture was complete after 3 days, while the uncatalyzed reaction had only reached 50% conversion after two weeks. Thus, the dynamic disulfide mixture was able to catalyze the aza-Cope rearrangement. We could not quantitatively monitor the disappearance of the substrate by ^{1}H-NMR, as it formed broad signals in presence of the catalyst. However, analysis by HPLC revealed that the rate at which the substrate disappeared corresponded well to the rate at which the product appeared (Figure 4.5). Hence, there was no significant build-up of any intermediate, indicating that the aza-Cope rearrangement remained the rate-determining step and that the subsequent hydrolysis was still fast.

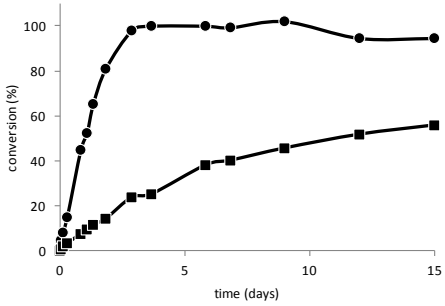


Figure 4.4 Appearance of product **5** in a reaction mixture starting from 2.00 mM **1** as a function of time in the absence (■) and presence (●) of a DCL made from building block **7** (8.00 mM in 100 mM phosphate buffer in D_2O, pD = 8.0, monitored by ^{1}H-NMR).

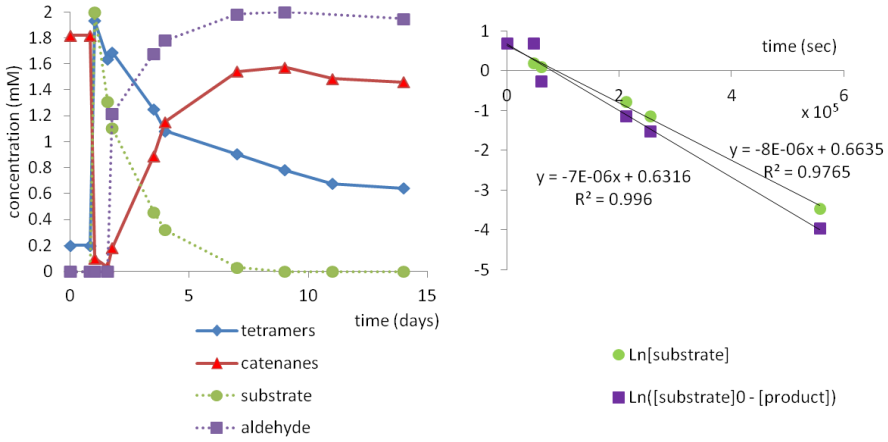


Figure 4.5 Left: Variation of the composition of a dynamic combinatorial library made from **7** upon addition of substrate **1** at day 1. Right: Rate of disappearance of substrate **1** and rate of appearance of dimethylamine product in the presence of a dynamic combinatorial library made from **7** (monitored by HPLC). Conditions: [**1**] = 2.00 mM in borate buffer (50 mM in D_2O pD 8.0), [**7**] = 8.00 mM.

We also investigated the effect of the TSA on the rate of the catalyzed reaction. As expected, the TSA acted as a competitive inhibitor of the aza-Cope rearrangement. Figure 4.6 shows how the rate of the catalyzed reaction diminishes in the presence of increasing amounts of TSA. The inhibitory effect of the TSA is relatively small – some catalysis is still taking place in the presence of 10 equivalents of TSA – suggesting that it has an unexpectedly weak affinity for the catalyst. This was further confirmed in a templating experiment in which a DCL made from **7** was exposed to a 1:1 mixture of substrate **1** and TSA **6**, where we monitored the product distribution of the network immediately after adding the templates, before any **1** had reacted. This experiment resulted in the amplification of the isomeric tetramers of **7** with a ratio that matched the one obtained when only the substrate was used as a template, but was different from the ratio obtained when using only the TSA (Figure 4.7). Taken together, these results suggest that the substrate binds the tetramers more strongly than the TSA. The fact that we nevertheless obtain catalysis indicates that the TSA is not a perfect model for the real transition state.

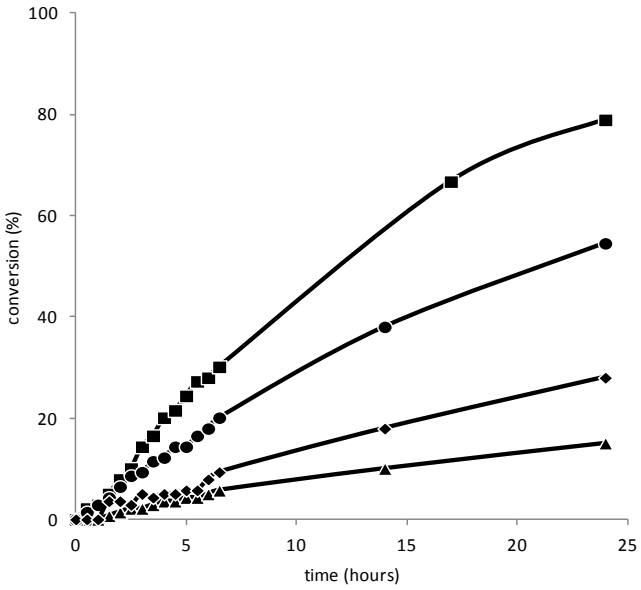


Figure 4.6 Kinetics of the aza-Cope rearrangement in absence of catalyst **9** (▲); in presence of 1.0 equiv of **9** (■); in presence of 1.0 equiv of **9** and 1.0 equiv of TSA **6** (●) and in the presence of 1.0 equiv of **9** and 10 equiv of TSA (♦) as inhibitor. Conditions: 2.00 mM of substrate **1** in 50 mM borate buffer, pH 8.0.

Three consecutive additions of substrate **1** (respect to **9**) to a DCL made from building block **7** over a period of 3 weeks resulted in nearly full conversion of the substrate, evidencing that the catalyst is capable of turnover (Figure 4.8). Other reaction substrates of the same family as **1** were also tested and comparable catalytic effects were obtained (Figure 4.9). Only small differences in terms of templating and catalytic effects can be observed when comparing the results obtained for substrates **10** (Figure 4.9, top) and **11** (Figure 4.9, bottom). The library composition in presence of substrate **10** shows a ratio catenanes/tetramers of about 1/3 while in presence of **11** virtually only tetramers are present. This difference is presumably due to a preferred binding of the tetramers to substrate **11** over **10**. The fact that the aza-Cope rearrangement of **11** is more efficiently catalyzed than the corresponding reaction of **10** suggests that the transition state of the former reaction is more efficiently stabilized than that of the latter.

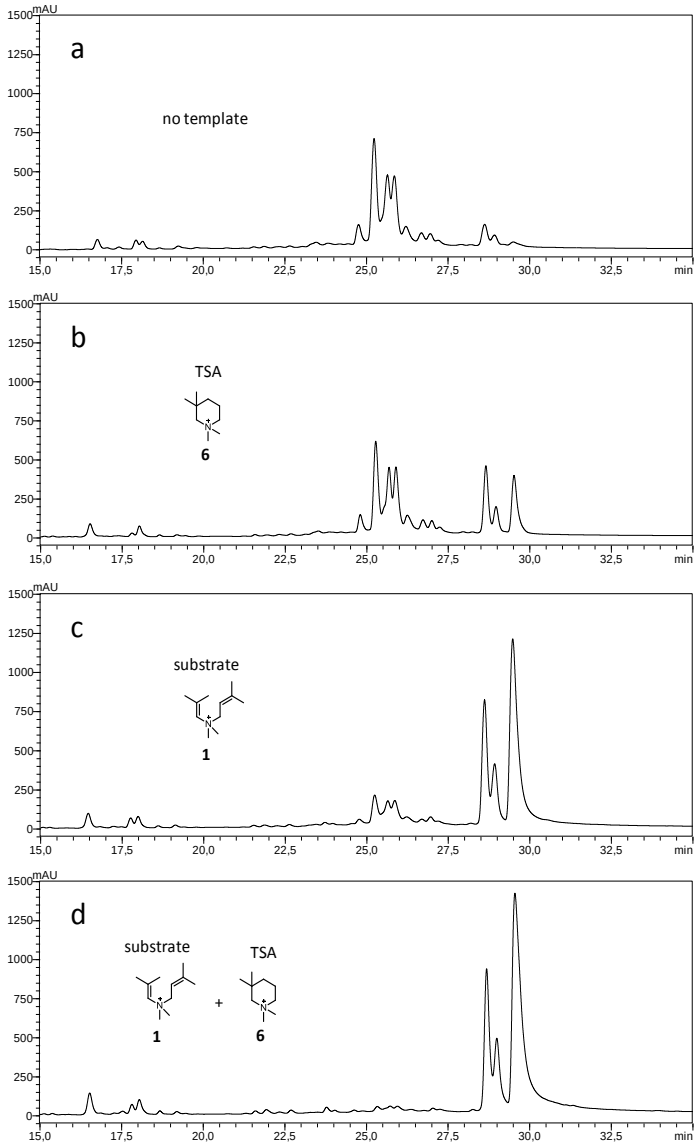


Figure 4.7 HPLC chromatograms showing the composition of a DCL made from 8.00 mM **7** in borate buffer 50 mM pH 8.0 a) in the absence of template, and in the presence of: b) 2.00 mM TSA **6**; c) 2.00 mM substrate **1**; d) an equimolar mixture of TSA and substrate **1** (2.00 mM each). The disulfide exchange reaction was frozen 1 minute after addition of template by acidifying the solutions to pH 3 with aqueous HCl, ensuring that the library compositions capture the stage where the concentrations of substrate and TSA are equal.

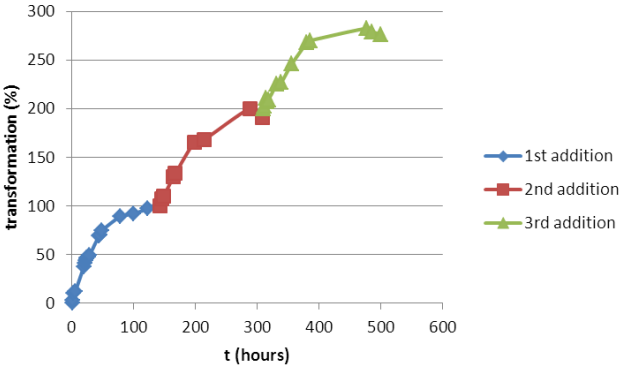


Figure 4.8 Transformation of substrate **1** as a function of time in the presence of a dynamic library made from building block **7**. Transformation was determined by monitoring the amount of formed dimethylamine by ^{1}H-NMR. Conditions: [**9**] = 2.00 mM in borate buffer (50 mM in D_2O pH 8.0). Dimethylacetamide was used as internal standard. [**1**], first addition: 1.48 mM (rhombi), second addition: 2.00 mM (squares), third addition: 2.00 mM (triangles).

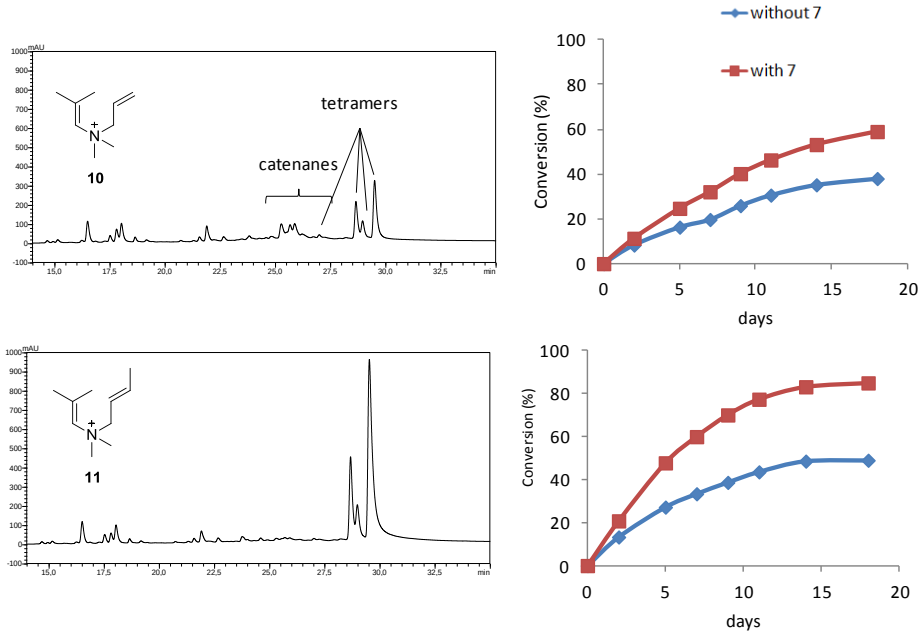


Figure 4.9 Product distribution of a dynamic combinatorial library made from **7** in presence of templates **10** (top) and **11** (bottom) and their corresponding conversion as a function of time compared the conversion in absence of **7**. The conversion was determined by monitoring the amount of dimethylamine by ^{1}H-NMR. Acetonitrile was used as internal standard. (b) Conditions: [template] = 2.00 mM, [**7**] = 8.00 mM, in borate buffer 50 mM in D_2O, pD 8.0.

4.5 Effect of the aza-Cope rearrangement on the composition of the dynamic combinatorial library

While the aza-Cope rearrangement is catalyzed in the small dynamic disulfide network, the aza-Cope rearrangement, in turn, also influences the product distribution of the disulfide network. We monitored the distribution of the disulfide products over the course of the reaction. In the absence of aza-Cope substrate **1** the mixture was dominated by catenanes **8** and its composition did not change in the course of 40 days (Figure 4.10a, solid lines). Introducing substrate **1** resulted in a rapid re-equilibration in favor of tetramers **9**, as shown in Figure 4.10b. As **1** was consumed in the course of the aza-Cope rearrangement, the disulfide composition gradually reverted back to catenanes **8**. The products of the catalyzed reaction did not have a detectable effect on the library distribution, as shown by addition of dimethylamine and 2,2-dimethylpent-4-enal to a dynamic library made from **7** (Figure 4.10a, dotted lines). The overall behavior of the system is summarized in Figure 4.11.

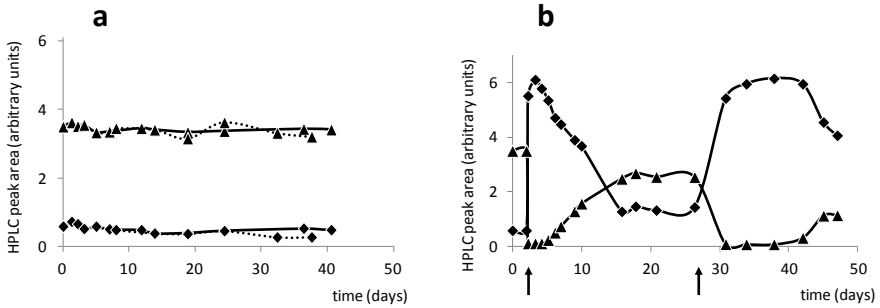


Figure 4.10 Kinectic profiles. a), The disulfide network in the absence of **1** (solid lines) consists of a constant concentration of catenanes **8** (▲) and tetramers **9** (♦) which is unaffected by the presence of the products of the aza-Cope reaction: dimethylamine and 2,2-dimethylpent-4-enal (dotted lines). b), Change in composition of the dynamic disulfide network over the course of the aza-Cope rearrangement, showing the rapid conversion of catenanes **8** (▲) into tetramers **9** (♦) upon adding substrate **1** at day 2 followed by slow return back towards **8** as substrate **1** is converted into product **5**. Further **1** was added at t=24 days. Arrows mark the points of addition of **1**.

The kinetics of the return towards the original disulfide distribution were slower than the re-equilibration upon the exposure to **1** and also somewhat slower than the catalyzed aza-Cope rearrangement (as seen by comparing Figure 4.4 with Figure 4.10b). We noticed

that, once **1** was consumed, tetramers **9** had the tendency to aggregate into fibres (cryo-TEM analysis is shown in Figure 4.12).

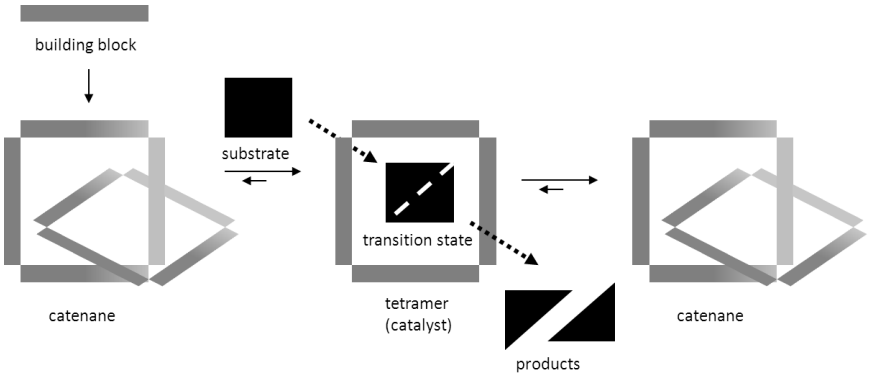


Figure 4.11 A solution of building blocks gives rise to a dynamic combinatorial library where the dominant library member changes from the initial catenane to the catalytic tetramer upon addition of the reaction substrate. Once the substrate is consumed, the DCL shifts back towards the initial catenanes.

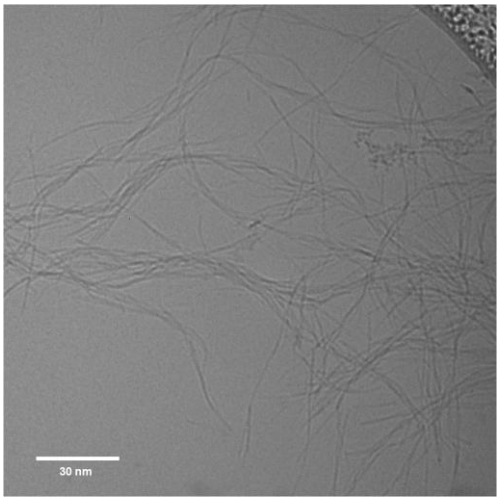


Figure 4.12 Cryo-TEM image of a solution of **9** showing self-assembled fibers.

We speculate that this aggregation process slows down the kinetics of disulfide exchange and prevents it from completely returning to the original composition. Unfortunately all attempts to prevent fiber formation in this system have failed so far. However, the system

remains dynamic, as introduction of more **1** after 24 days (Figure 4.10b) induced the re-emergence of the tetramers, and gave further conversion of **1** into aza-Cope products. These results suggested that the tetramers were able to catalyze the aza-Cope rearrangement. This conclusion was supported by experiments in which we used completely oxidized, and therefore no longer dynamic, disulfide mixtures that were dominated by either the catenanes **8** or the tetramers **9**, and which showed that the aza-Cope rearrangement was accelerated by **9**, but not by **8** (Figure 4.13). This outcome rules out the involvement of partially oxidized library members in catalysis.

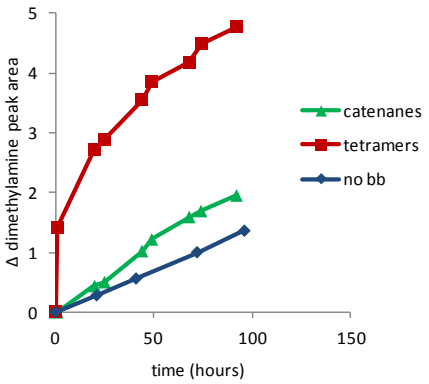


Figure 4.13 ^{1}H-NMR data showing the increase of peak area of the signal corresponding to dimethylamine resulting of an aza-Cope rearrangement of **1** in a static solution of a) catenanes **8** (triangles); b) tetramers **9** (squares); and c) in absence of **7** and its oxidation products (rhombi). Conditions: [1] = 2.00 mM, [7] = 8.00 mM in borate buffer 50 mM pH = 8.0.

To better understand the mechanism of the catalyzed reaction, a study on the reaction order of substrate and catalyst was performed. The rate constants of the catalyzed and non-catalyzed reactions were determined and compared, confirming the catalytic properties of **9**.

4.6 Determination of the reaction order in substrate and rate constants

A reaction that is first order in substrate will obey the following kinetics:

$[substrate]_t = [substrate]_0 e^{-kt}$

where $[substrate]_0$ and $[substrate]_t$ equal the concentration substrate at time = 0 and time = t, respectively, and *k* equals the rate constant.

Hence, $\ln[substrate]_t = \ln[substrate]_0 - kt$

Thus, when plotting $\ln[substrate]_t$ versus time, a reaction that is first order in substrate will give a straight line with slope *-k*.

Substrate concentrations were determined by NMR (using acetonitrile as an internal standard) or by HPLC. For the assesment of conversion of substrate by HPLC, it was assumed that there was no conversion just after the substrate was introduced (first datapoint corresponds to a library after a few seconds), so that the peak area of the substrate would correspond to the 2.0 mM concentration that was initially prepared.

The results of the analysis of the catalyzed and the uncatalyzed aza-Cope rearrangement are shown in Figure 4.14, showing that both reactions are first order in substrate **1**. The (apparent) rate constants for the catalyzed and the uncatalyzed reactions are 7.4 x 10^{-6} s^{-1} and 1.8 x 10^{-6} s^{-1}, respectively (obtained at 20 °C; Raymond reported a rate constant for the uncatalyzed reaction of substrate **1** of 6.3 x 10^{-5} s^{-1} , obtained at 50 °C).[35]

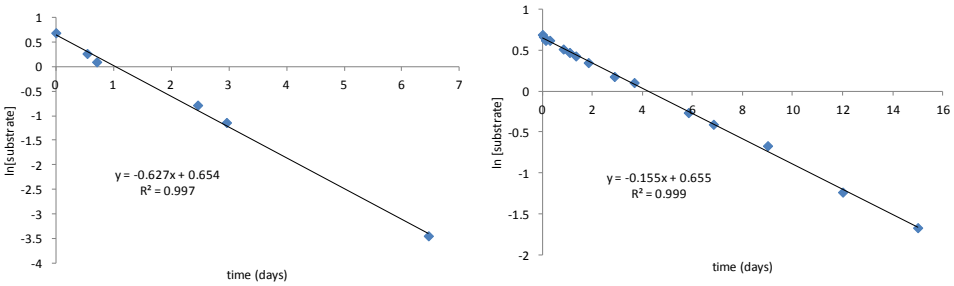


Figure 4.14 Plots of ln[substrate] versus time for the reaction of **1** (2.00 mM) in the presence of a small dynamic molecular network made from 8.00 mM **7** (left) and for the uncatalyzed reaction (right). Rate constants were found to be 7.4 x 10^{-6} s^{-1} for the catalyzed reaction and 1.8 x 10^{-6} s^{-1} for the uncatalyzed reaction. All reactions were performed in 100 mM phosphate buffer pH 8.0. The reaction of the substrate was monitored by ^{1}H-NMR for the uncatalyzed reaction and by HPLC for the catalyzed reaction, as the binding of the substrate to the catalyst broadens ^{1}H-NMR signals hampering their accurate integration.

4.7 Determination of the reaction order in catalyst

The kinetics of the catalyzed aza-Cope rearrangement may be described as:

$$d[\mathbf{1}]/dt = -k_0\,[\mathbf{1}] - k_{cat}[\text{complex}] \qquad \text{(Eq. 1)}$$

where k_0 is the rate constant for the uncatalyzed reaction; k_{cat} is the rate constant for reaction of the substrate-catalyst complex. Complex formation may be described as,

$$[\text{complex}] = K\,[\mathbf{1}]\,[\mathbf{9}]^{p} \qquad \text{(Eq. 2)}$$

where p is the stoichiometry of **9** in the complex.

Substituting Eq. 2 into Eq. 1 gives:

$$d[\mathbf{1}]/dt = -k_0\,[\mathbf{1}] - k_{cat}K\,[\mathbf{1}]\,[\mathbf{9}]^{p} \qquad \text{(Eq. 3)}$$

Which may be rewritten as:

$$\log (d[\mathbf{1}]/dt) = \log (-k_0\,[\mathbf{1}] - k_{cat}K\,[\mathbf{1}]\,[\mathbf{9}]^{p}) \qquad \text{(Eq. 4)}$$

When only considering the contribution from the catalyzed reaction:

$$\log (d[\mathbf{1}]/dt)_c = \log (-k_{cat}K\,[\mathbf{1}]\,[\mathbf{9}]^{p}) = \log (-k_{cat}K\,[\mathbf{1}]) + p \log [\mathbf{9}] \qquad \text{(Eq. 5)}$$

Thus, plotting $\log (d[\mathbf{1}]/dt)_c$ versus $\log [\mathbf{9}]$ should give a straight line with a slope equal to the order in catalyst p.

We have determined the initial rate of the reaction at four different catalyst concentrations. A mother solution 8.00 mM of **7** in borate buffer (50 mM in D_2O pH 8.0) was prepared and allowed to partially oxidize to give a dynamic mixture of disulfides. Five different solutions were prepared by adding different proportions of the mother solution to reach a final concentration of 0, 2.00, 4.00, 6.00 and 8.00 mM of **7**. To these five solutions, **1** was added to obtain a concentration of 2.00 mM and the formation of dimethylamine was monitored by ^{1}H-NMR. For each solution, the concentration of dimethylamine was plotted against time to obtain the initial rate of reaction from the slopes of these plots (Figure 4.15, top). The rate of the background reaction was then subtracted from the observed rates of the reactions in the presence of catalyst and the logarithm of the thus corrected rates were plotted against the logarithm of the catalyst concentrations, assuming that all available **7** is converted quantitatively into catalyst **9** (Figure 4.15, bottom). From the slope of the plot the order in catalyst was found to be 1.04.

From Eq. 5 and making use of the same plot in Figure 4.15 the equilibrium constant K was resolved. Substituting the value of k_{cat} found in the previous section (4.6) a value of K =

$1.2 \cdot 10^6\ M^{-1}$ was obtained. This high value of the equilibrium constant indicates a very high proportion of substrate associated to the catalyst and is in agreement with the fast response of the library observed upon addition of the substrate.

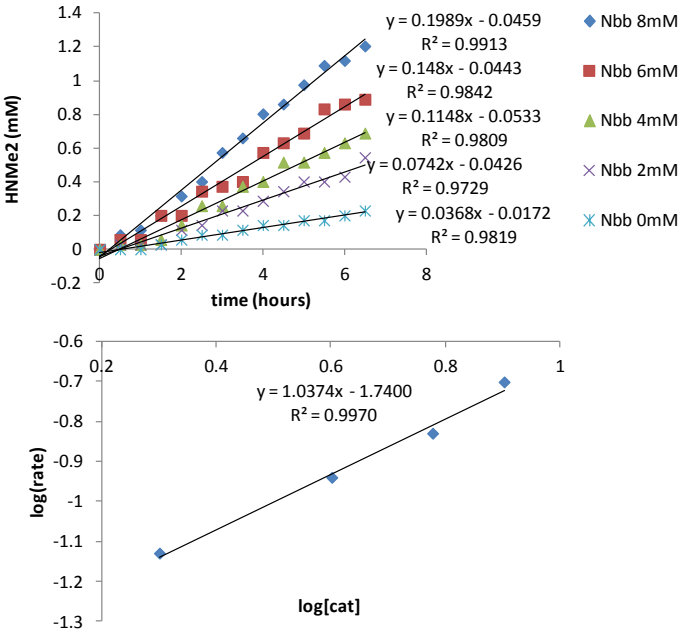


Figure 4.15 Top: Increase of dimethylamine concentration as a function of time for five different solutions with a concentration of 0 (stars), 0.25 (crosses), 0.50 (triangle), 0.75 (squares) and 1.00 equiv (rhombi) of catalyst **9** relative to substrate **1**. Conditions: [**1**] = 2.00 mM in borate buffer (50 mM in D_2O pH 8.0); CH_3CN was used as internal standard for ^{1}H-NMR integration. Bottom: log-log plot of the initial rate of the aza-Cope rearrangement for the four solutions containing different catalyst concentration.

4.8 Conclusions

We have demonstrated that screening DCLs using both starting material and TSA enables the identification of a dynamic molecular network that responds to the substrate of a reaction by transiently producing its catalyst. After completion of the reaction the mixture returns towards its original composition. This dynamic molecular network approach to catalysis, with in-situ and on-demand formation and consumption of the catalyst, opens new possibilities for control over synthetic catalytic systems. For example, several catalytic systems could be assembled sequentially to transform a substrate into a product in separate catalytic steps.

4.9 Experimental

Reagents and solvents

All reagents and solvents were obtained from commercial sources and used without further purification unless specified otherwise. Dry solvents were obtained from an MBraun SPS-800 solvent purification system.

NMR analysis

NMR spectra were obtained on a Varian AS 400 MHz instrument. ^{1}H chemical shifts are reported as δ in ppm relative to residual protonated solvent resonances. ^{13}C chemical shifts are reported as δ in ppm and measured relative to solvent references. Coupling constants are reported in Hertz.

HPLC analysis

Analytical HPLC was carried out on Hewlett Packard 1050 or 1100 systems coupled to UV detectors and the data were processed using HP Chemstation software. Separations were performed on a reversed phase Zorbax Eclipse XDB-C8 column (4.6 x 150 mm, 5 µm particle size). Aliquots of 3 µL of library solution were injected. Doubly distilled water, HPLC-S-grade acetonitrile from Biosolve and formic acid were used to prepare the eluents:
eluent A = water + 5.0 % acetonitrile + 0.10 % formic acid
eluent B = acetonitrile + 5.0 % water + 0.10 % formic acid
Chromatography was performed at 45 °C using UV detection at 260 nm and a constant flow rate of 1.00 mL / min. The HPLC analysis method was as follows:

Time (min)	Eluent A (%)	Eluent B (%)
0	80	20
5	80	20
27	10	90
35	10	90
38	80	20
43	80	20

LC-MS analysis

For the LC-MS measurements an Accela High Speed LC system (ThermoFisher Scientific, Courtaboeuf, France) was coupled to a LTQ-Fleet Ion Trap Mass Spectrometer. Mass spectra (negative ion mode) were obtained using the following conditions: sheath gas flow rate 8, aux. gas flow rate 2, sweep gas flow rate 2, ionization spray voltage 3.50 kV, capillary temperature 275 °C, capillary voltage -21 V, tube lens -120.60 V.

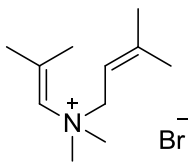
The LC method was the same as that used for HPLC. The flow was split after the LC to allow 0.30 mL / min to enter the mass spectrometer.

Cryo-TEM protocol

A small drop of suspension was placed on a Quantifoil 3.5/1 holey carbon coated grid. Blotting and vitrification in ethane was done in a Vitrobot (FEI, Eindhoven, The Netherlands). The grids were observed in a Philips CM120 cryo-electron microscope operating at 120 kV with a Gatan model 626 cryo-stage. Images were recorded under low-dose conditions with a slow-scan CCD camera.

Synthetic Procedures

*N,N,3-trimethyl-N-(2-methylprop-1-enyl)but-2-en-1-aminium bromide (***1***)* was prepared following a modified literature procedure:[34]



Preparation of *N,N*,2-trimethylprop-1-en-1-amine: dimethylamine was obtained by distillation from a 40 % dimethylamine solution in water. Freshly distilled isobutyraldehyde (5.8 g, 80 mmol) and dimethylamine (4.0 g, 88 mmol) were added to a cooled mixture of 10 mL *m*-xylene and K_2CO_3 (6.00 g, 43.4 mmol) in an autoclave. The mixture was stirred and heated to 100 °C for 4 hours. After distillation using a vigreux column 11.2 g (32 % yield) of *N,N*,2-trimethylprop-1-en-1-amine was obtained (b.p. 89 °C) as a colorless liquid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.21 (s, 1H, =CH), 2.28 (s, 6H, $N(CH_3)_2$), 1.58 (s, 3H, CH_3), 1.50 (s, 3H, CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 136.9 (=CH), 120.6 (C_{quat}), 45.3 ($N(CH_3)_2$), 22.2 (CH_3), 17.1 (CH_3).

A two-necked round bottomed flask was placed in a bath at -2 °C and charged with 2.0 mL of dry acetonitrile and 1-bromo-3-methylbut-2-ene (1.35 mL, 11.3 mmol) under a N_2

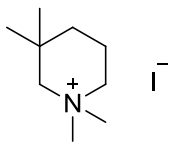
atmosphere. *N,N*,2-trimethylprop-1-en-1-amine (0.50 mL, 3.8 mmol) was then slowly added using a syringe. The solution was left stirring at -2 °C overnight. The acetonitrile was evaporated under vacuum and the remaining oil was thoroughly washed with previously cooled (-20 °C) dry ether (5 x 5 mL). The product was obtained as a pale yellow oil (0.81 g, 87 % yield) that solidified at -20 °C. ^{1}H-NMR (400 MHz, D_2O): δ 5.70 (s, 1H), 5.28 (t, *J* = 8.0Hz , 1H), 3.97 (d, *J* = 8.0Hz, 2H), 3.12 (s, 6H), 1.86 (d, *J* = 1.2Hz, 3H), 1.73 (s, 3H), 1.68 (d, *J* = 1.3Hz, 3H), 1.65 (s, 3H). ^{13}C-NMR (100 MHz, CD_3OD): δ 146.8 (C_{quat}), 134.6 (C_{quat}), 126.6 (=CH), 109.0 (=CH), 63.8 (CH_2), 51.4 ($N(CH_3)_2$), 23.8, 23.0, 16.6, 16.2 (4 x CH_3). HR-MS (ESI+ of M^+): *m/z* calcd for $C_{11}H_{22}N$ 168.1747, found 168.1754.

*Aza-Cope substrates **10** and **11** were synthesized following the procedure described above for **1** using allyl bromide and (E)-4-bromo-2-butene as substrates.*

Analytical data for **10:** obtained as a pale yellow oil (82% yield). ^{1}H-NMR (300 MHz, D_2O): δ 5.88 (m, 1H), 5.77 (s, br, 1H), 5.55 (dd, *J* = 17Hz, *J* = 1.2Hz, 2H), 4.01 (d, *J* = 6.0Hz, 2H), 3.17 (s, 6H), 1.88 (s, 3H), 1.69 (s, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 136.7 (=CH), 129.3 (=CH), 128.6 (C_{quat}), 124.9 (=CH_2), 69.6 (CH_2), 54.4 ($N(CH_3)_2$), 25.6, 19.4 (2 x CH_3). HR-MS (ESI+ of M^+): *m/z* calcd for $C_9H_{18}N$ 140.1434, found 140.1433.

Analytical data for **11:** obtained as a yellow oil (76% yield). ^{1}H-NMR (300 MHz, D_2O): δ 6.04 (m, 1H), 5.72 (s, br, 1H), 5.53 (m, 1H), 3.92 (d, J = 3.1Hz, 2H), 3.13 (s, 6H), 1.86 (s, 3H), 1.69 (s, 3H), 1.67 (d, *J* = 4.5Hz, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 135.2 (=CH), 128.8 (=CH), 128.3 (C_{quat}), 116.5 (=C-), 69.3 (CH_2), 53.9 ($N(CH_3)_2$) 25.2, 19.0, 18.3 (3 x CH_3). HR-MS (ESI+ of M^+): *m/z* calcd for $C_{10}H_{20}N$ 154.1590, found 154.1589.

*1,1,3,3-Tetramethylpiperidinium iodide (**6**)*



3,3-Dimethylpiperidine (0.50 g, 4.4 mmol) was dissolved in 4.0 mL of MeOH. $KHCO_3$ (0.66 g, 6.6 mmol) was added and, after cooling the mixture in an ice bath, MeI (1.87 g, 13.2 mmol) was added dropwise while stirring. The reaction mixture was left at r.t. for 4 days. The solvents were removed under vacuum and the solid was dissolved in 4 mL CH_3Cl. The remaining solids were filtered and then the CH_3Cl was evaporated. The solid was dissolved in 12 mL of refluxing isopropanol to which the minimum amount of EtOH was added to improve the solubilization. The solution was left to cool down and 0.68 g (2.5 mmol, 57 %

yield) of product was obtained as white needles. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 3.69 (dd, *J* = 9.1Hz, 14.8, 2H), 3.52 (d, *J* = 9.4Hz, 2H), 3.48 (s, 6H), 2.04 – 1.92 (m, 2H), 1.68 – 1.58 (m, 2H), 1.15 (s, 6H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 72.0 (CH_2), 62.6 (CH_2), 54.7 ($N(CH_3)_2$), 33.8 (CH_2), 31.29 (C_{quat}), 28.62 (2 x CH_3), 18.12 (CH_2). m.p. 316.1 - 318.3 °C. HR-MS (ESI+ of M^+): *m/z* calcd for $C_9H_{20}N$ 142.1590, found 142.1586.

*Dithiol building blocks **7**,[37] **12**,[38] **13**,[39] were prepared as described previously.*

Templating experiments corresponding to Figure 4.3 (top, middle, bottom)

The following procedures were performed in a glove box to exclude oxygen. Borate buffer (50 mM pH 8.0) was used for preparing solutions and was previously deoxygenated by purging with N_2 for 1 h. The libraries were stirred in closed vials using magnetic stirrers.

A solution of 8.00 mM of **7** was prepared in borate buffer. A controlled oxidation of **7** (monitored by HPLC) was performed by adding aliquots of a 200 mM solution of sodium perborate until 2/3 of the starting material was oxidized.

Three samples of 1.0 mL were taken from this solution and combined with:

sample a: 16 µL borate buffer (Figure 4.3, top)

sample b: 16 µL of a 125 mM solution of **6** in borate buffer (Figure 4.3, middle)

sample c: 16 µL of a 125 mM solution of **1** in borate buffer (Figure 4.3 bottom)

After 12 h, 15 µL samples of the templated solutions were transferred into vials that were tightly sealed before being removed from the glove box and analyzed by HPLC.

The presence of 0.1% of formic acid in the mobile phase renders the HPLC sample non-dynamic as thiolates will be protonated. This ensures that there is effectively no re-equilibration of the library during the analysis.

Catalysis experiments corresponding to Figures 4.4 and 4.10 (a,b)

The catalytic behavior of **9** was monitored by comparing the rate of increase of the dimethylamine ^{1}H-NMR signals in a sample in the presence and absence of the dynamic mixture made from **7** (Figure 4.16). For that, 1.0 mL of an 8.00 mM solution of **7** in borate

buffer (50 mM pD 8.0, prepared with D_2O)[a], was transferred to a first NMR tube. A blank was prepared in absence of **7** in a second NMR tube. An aliquot of 16 µL of a solution of 125 mM of **1** in borate buffer was added to each of the NMR tubes. Several NMR spectra were recorded during the following weeks for both samples and the results were plotted as change of ^{1}H-NMR peak area of **5** (normalized to the peak area of CH_3CN[a]) vs. time.

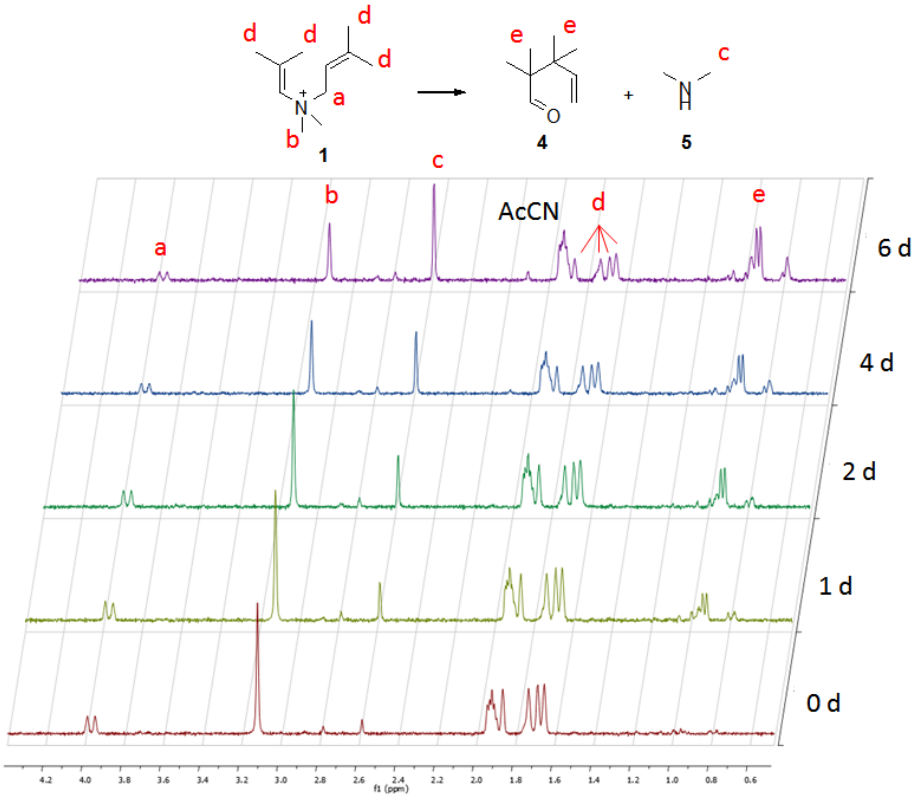


Figure 4.16 Aza-Cope rearrangement monitored by ^{1}H-NMR at t = 0, 1, 2, 4 and 6 days. The peaks corresponding to the substrate (a, b, d) decrease while the ones from the products (c, e) increase with time. Conditions: [**1**] = 2.00 mM in borate buffer (50 mM in D_2O pH 8.0). CH_3CN was used as internal standard.

The change in the composition of the dynamic disulfide network during the catalytic process was monitored by HPLC analysis of a mixture formed by a partial oxidation of **7** (as described above) in presence (Figure 4.10b), and absence of **1** (Figure 4.10a).

[a] Borate buffer contained 15 µL of CH_3CN / 100 mL buffer. The relative intensities of the dimethylamine peaks referenced to the peaks of CH_3CN were monitored. This procedure allows the increase of dimethylamine peak area to be compared between two different ^{1}H-NMR samples.

Catalysis experiments with non-dynamic catenanes and tetramers (Figure 4.13)

A solution containing mainly catenanes was prepared by dissolving **7** (8.00 mM solution in 50 mM borate buffer pH 8.0) and letting it oxidize for 10 days to form disulfides until no more dithiol building block was present, reaching therefore a non-dynamic state. Under these conditions the library yielded mostly catenanes (94% as estimated from HPLC peak areas). To obtain a disulfide mixture containing essentially tetramers, substrate **1** was added to reach a concentration of 2.00 mM in a solution of 8.00 mM **7**. This mixture was exposed to oxygen from the atmosphere. After three weeks the mixture still contained 90% tetramers (as estimated from the HPLC peak areas) while no remaining substrate was found as it had fully reacted to give the aza-Cope products. The disulfides in this solution were also in a static state due to the absence of thiolate anion.

Three different samples were prepared by adding 5.6 µL of a solution of substrate **1** (125 mM) to:

a) 280 µL of the solution containing mainly catenanes (Figure 4.13, green triangles);

b) 280 µL of the solution containing mainly tetramers (Figure 4.13, red squares);

c) 280 µL of borate buffer in the absence of **7** or its oxidation products (Figure 4.13, blue rhombi).

Additionally, 200 µL of D_2O were added to each sample for locking purposes. The increase of the signal corresponding to dimethylamine was monitored during the first days of the aza-Cope rearrangement by ^{1}H-NMR using water presaturation. The difference in the rearrangement rate between the sample with tetramers and the one with catenanes suggests that **9** is the catalytically active species.

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Chapter 5

Molecular Communication between Dynamic Molecular Networks

5.1 Introduction

Compartmentalization is one of the characteristics that, at a higher or lower degree of complexity, all living organisms share. Living systems are constituted by biologically incompatible chemistries or processes that need to be isolated by membranes: at cellular level, lipid membranes separate the cytoplasm from the surroundings.[1] Yet for cells to survive, it is essential to sense and respond to changes in the environment via input, output and processing components which are often proteins built from simple modular structures.[2,3] Moreover, cell-cell communication is critical to interactive communities such as most bacteria species.[4-6] Through molecular engineering, scientists have developed excellent examples of *in-vivo* signaling circuits like autoregulatory systems,[7,8] oscillators[9] or toggle switches[10-12] with the aim of programming new cell behaviors. Nonetheless, interactions between synthetic communication components and the chemical compounds in the hosts make it difficult to design and study new signaling systems.[13] Therefore, further research on signaling strategies at molecular level using uncomplicated chemical environments is needed.[14] From our work with complex systems[15-18] we learnt that dynamic combinatorial chemistry (DCC) is a powerful tool for preparing networks of interconverting chemical species starting from simple building blocks. Making use of the possibilities of DCC to yield combinatorial outputs responsive to environmental changes, we engineered *in-vitro* systems of separated molecular arrangements capable of communicating. The setups were conceived as simple bidirectional communication modules where signaling properties would be under control and therefore easy to study. Hydrazone and disulfide based DCLs were used to prepare such systems. Initial experiments aimed to achieve intercommunication between liposomes via hydrazone exchange were also performed.

5.2 Hydrazone-based libraries

Triphasic systems were designed to mimic the alternated aqueous-organic-aqueous nature of the environment when travelling from the cytoplasm via the cell membrane to the extracellular space which contained two aqueous phases (semi-system a, semi-system b) separated by an organic phase. In each of the aqueous phases, a combination of

hydrazones formed by condensation of hydrazides with aldehydes was prepared as a representation of the intra-/extracellular chemical composition. The communication between aqueous phases was achieved by the transfer through the organic phase of the aldehydes that were formed by partial hydrolysis of the initial hydrazones. These aldehydes, which behaved as signals, travelled through the organic phase and interacted with the hydrazones present in the opposite semi-systems to induce a reequilibration of the hydrazone composition. The permanent charge existing on the hydrazide moieties prevented either the hydrazides or the corresponding hydrazones from diffusing across the apolar organic phase, allowing each of the two semi-systems to conserve their own identity defined by the hydrazide moiety present in them. When both semi-systems were connected, their composition slowly shifted until the hydrazone ratio in both aqueous phases reached a constant value, meaning that a thermodynamic equilibration of the two aqueous semi-systems had been achieved even when the different hydrazones were not in direct contact. These types of dynamic networks able to reach equilibration through a selective barrier are completely new and complementary to the already known one-phase[19] or even two-phase[20-22] combinatorial libraries where only one aqueous bulk containing all the hydrophilic species is present. They are also related to some of the few examples found on molecular transport through an organic phase in dynamic combinatorial chemistry.[23-25]

To test whether this idea of communication between separated systems was feasible using DCC, a first experiment was conceived. Two different hydrazones **AY** and **BZ** were placed in semi-system 1a and 1b respectively (Figure 5.1, system 1). The hydrazones were prepared by mixing the corresponding hydrazides and aldehydes in formate buffer pH 4.0 to catalyze hydrazone exchange, and letting the mixtures reach a constant hydrazone concentration (24 hours). Both semi-systems were then connected by an organic solvent and their composition was monitored by HPLC for over a month (for the setup, see Figure 5.13 in the experimental section). During this period a re-equilibration of the composition of the two aqueous phases was observed. The entrance into semi-system 1a of aldehyde **Z** originally only present in semi-system 1b, led to the formation of the new hydrazone **AZ** by condensation of **Z** with **A**, which was provided by native hydrazone **AY**. It is important to remember that the reaction of hydrazone formation from a hydrazide and an aldehyde

is an equilibrium process. Therefore there is always a small amount of free aldehyde and hydrazide in solution, especially in aqueous media. As a consequence of the formation of **AZ**, aldehyde **Y** was released (displaced by **Z**) so its concentration in semi-system 1a increased accordingly. This step of aldehyde exchange can be regarded as the conversion of an input **Z** into an output **Y**. Aldehyde **Y** diffused consequently through the organic phase and reached semi-system 1b where it generated the new hydrazone **BY** by reaction with hydrazide **B** that is in equilibrium with hydrazone **BZ**. In this way, more aldehyde **Z** was released and the re-equilibration of the molecular network continued until the energy of the system reached a new minimum described by the final concentration balance of the four hydrazones as shown in Figure 5.2 (continuous lines).

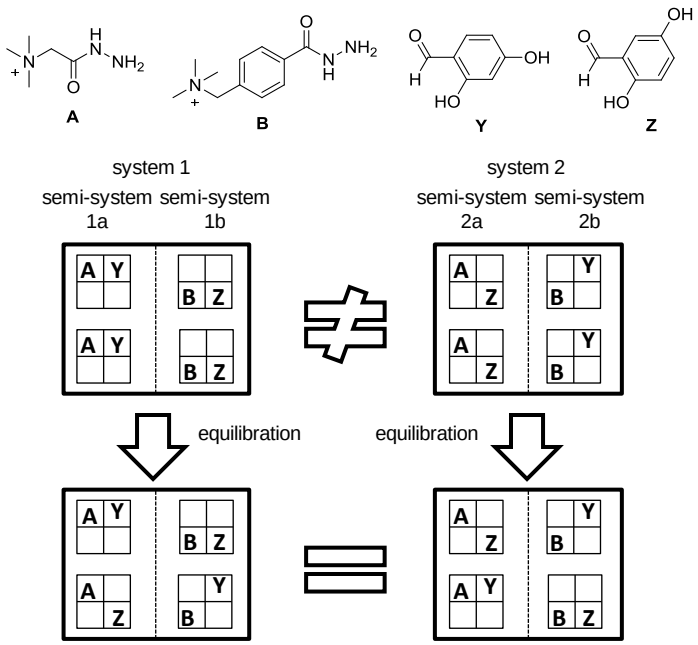


Figure 5.1 Schematic representation of the initial and final state of two initially different systems constituted by two aqueous hydrazone libraries connected through an organic solvent.

In a similar way, a system containing separated hydrazones **AZ** and **BY** (Figure 5.1, system 2) as starting compounds was prepared. The composition of its two semi-systems was monitored over time (Figure 5.2, dashed lines) and compared to system 1. The data shows that the concentrations of each of the four possible combinatorial compounds (**AY**, **AZ**, **BY**,

BZ) in the two different systems 1 and 2, when set up at different initial composition ratios (but keeping the concentration of the individual building blocks **A**, **B**, **Y**, **Z** constant), finally arrived to a common value. The decrease of the concentration of the initial hydrazones **AY** and **BZ** and the corresponding increase of **AZ** and **BY** in system 1 matched the increase of **AY** and **BZ** alongside with the decrease of **AZ** and **BY** in system 2 in such a way that the final composition of both systems was comparable. This thermodynamic equilibrium was reached after 5 weeks.

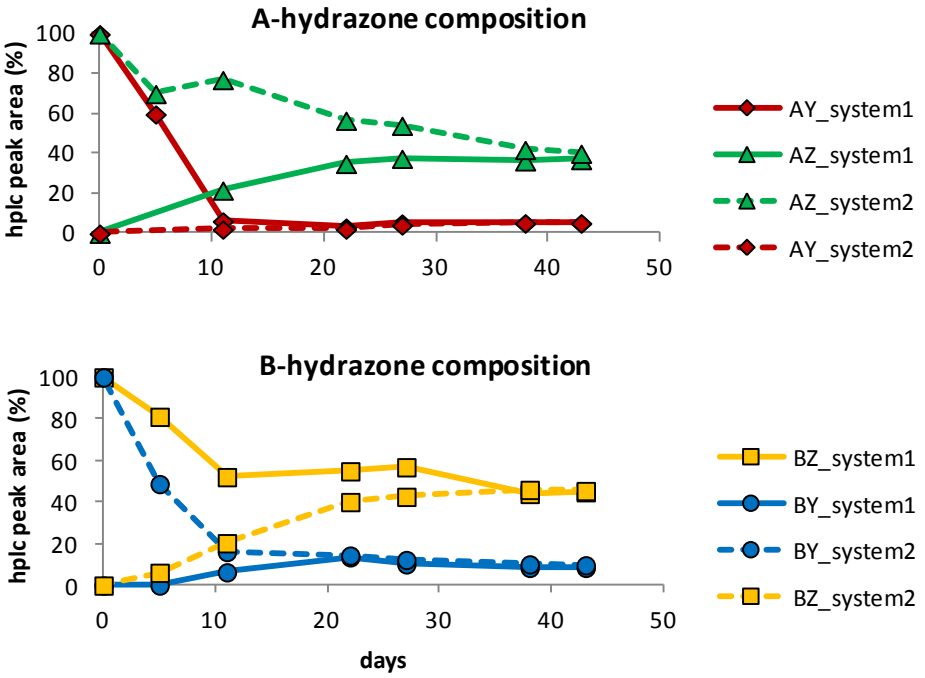


Figure 5.2 Composition shift of the hydrazones made from **A** (top) and the hydrazones made from **B** (bottom) expressed as percentage of total peak area of the chromatogram. The concentration of the initially present hydrazones in each semi-system decreased until a stable value was reached. Similarly, the concentration of the newly formed hydrazones rose to a stable value. The final concentration of all the hydrazones at equilibrium matched well for the two systems. Conditions: system 1, $[\mathbf{AY}]_o = [\mathbf{BZ}]_o = 7.50$ mM, system 2, $[\mathbf{AZ}]_o = [\mathbf{BY}]_o = 7.50$ mM . Aqueous phase: ammonium formate buffer 100 mM, pH 4.0; organic phase: EtOAc. See Figure 5.14 and Figure 5.15 for LC-MS analysis.

To rule out any background signal it was important to quantify the intensity of the signal in absence of key communication elements, for example measuring the amount of species

crossing the organic phase when no hydrazones were present in one of the chambers. A blank experiment where hydrazone **BZ** was present in semi-system 1b but no hydrazone was present in semi-system 1a revealed that the transfer of aldehyde **Z** to the adjacent semi-system was negligible when no hydrazone was present in one of the compartments. As seen in Figure 5.3, the concentration of **BZ** in semi-system 1b remained stable with time. Less than 1% of aldehyde **Z** was found in 1a after more than 1 month.

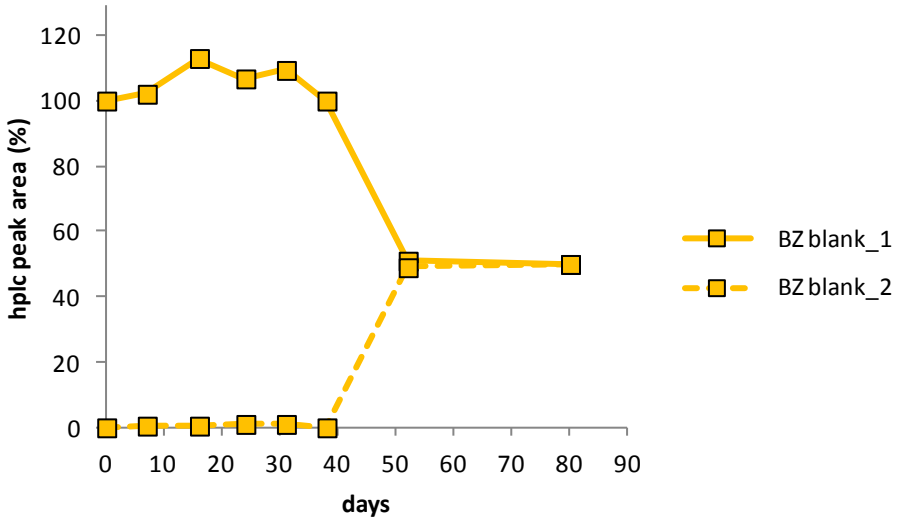


Figure 5.3 Variation of the composition of hydrazone **BZ** in the compartment initially containing **BZ** (continuous line) and the one initially empty (dashed line) expressed as percentage of total peak area of the chromatogram. Hydrazide **B** (7.50 mM) was added on day 39. The redistribution of aldehyde **Z** in the two compartments was complete within 12 days. [**BZ**] = 7.50 mM in ammonium formate buffer 100 mM, pH 4.0.

However, subsequent addition of hydrazide **B** to the empty semi-system triggered the redistribution of the aldehyde in both semi-systems and after 12 days a stable composition was reached. The presence of **B** drove the equilibrium to the formation of **BZ**, causing the signal transmission.

To further demonstrate the responsiveness of the setup, a system similar to the previous one but containing different hydrazones **BW** and **BZ** (Figure 5.4) was built. Initially, the new system was set to have the same ratio of **BW** and **BZ** in both semi-systems. Next, the system was perturbed by increasing the concentration of the hydrazide **B** in compartment

1a to a concentration two times higher than the initial one. The system answered by increasing the concentration of the hydrazones **BW** and **BZ** of semi-system 1a where **B** was added and as a result, the concentration of the hydrazones in the opposite compartment (semi-system 1b) decreased because of loss of **W** and **Z**. The system responded to this reduction in aldehydes by transferring more of them from 1b to 1a through the organic bulk. This effect produced a parallel decrease of the hydrazone reservoir and a subsequent increase of hydrazide concentration in 1b.

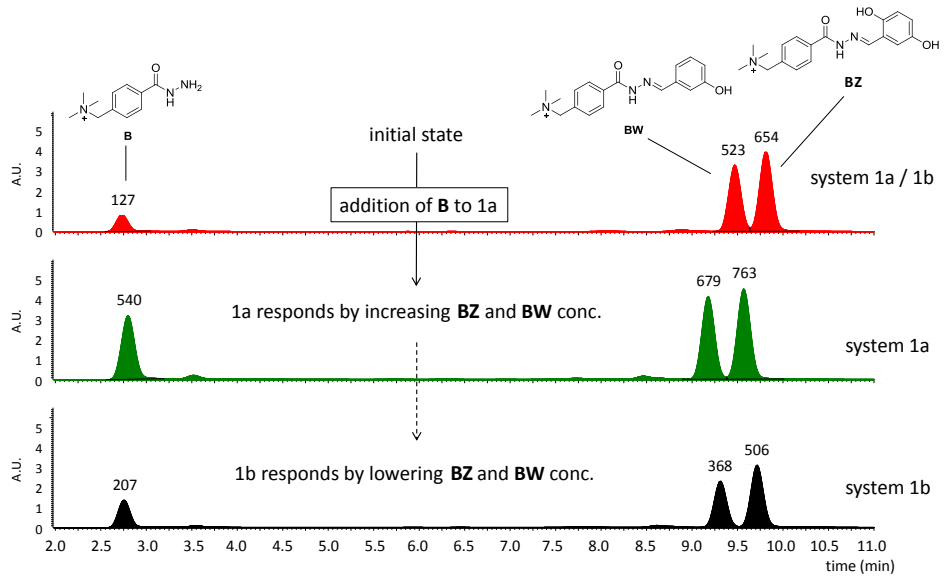


Figure 5.4 Initial state of an equilibrated hydrazone DCL of **BZ** and **BW** (top). After adding of **B** to semi-system 1a, it reacted with the free **Z** and **W** to increase the concentration of hydrazones **BZ** and **BW**. The addition of **B** also affected semi-system 1b which decreased its hydrazone concentration to release **Z** and **W**, that passed through the organic barrier, while molecule **B** remained in 1b. Conditions: $[\mathbf{BW}]_o = [\mathbf{BZ}]_o = 7.50$ mM = $\mathbf{B}_{added}$ in ammonium formate buffer (100 mM pH 4.0). See Figure 5.16 for LC-MS analysis.

The results show how a perturbation of one side of the molecular network led to a response of the other side which was in equilibrium with the first one through a connection that allows only a partial (but selective) molecular exchange.

A typical cellular communication system comprises a sensor (which converts a biological signal into an input) a regulator (which converts an input into an output) and an output interface (which converts an output into a biological response) (Figure 5.5). By using hydrazone exchange, a simplified version of this system has been created. The sensor, the

regulator and the output interface are now part of the same module: the hydrazone. The input-to-output response transduction is now defined by the equilibrium constants of the hydrazones involved in the exchange.

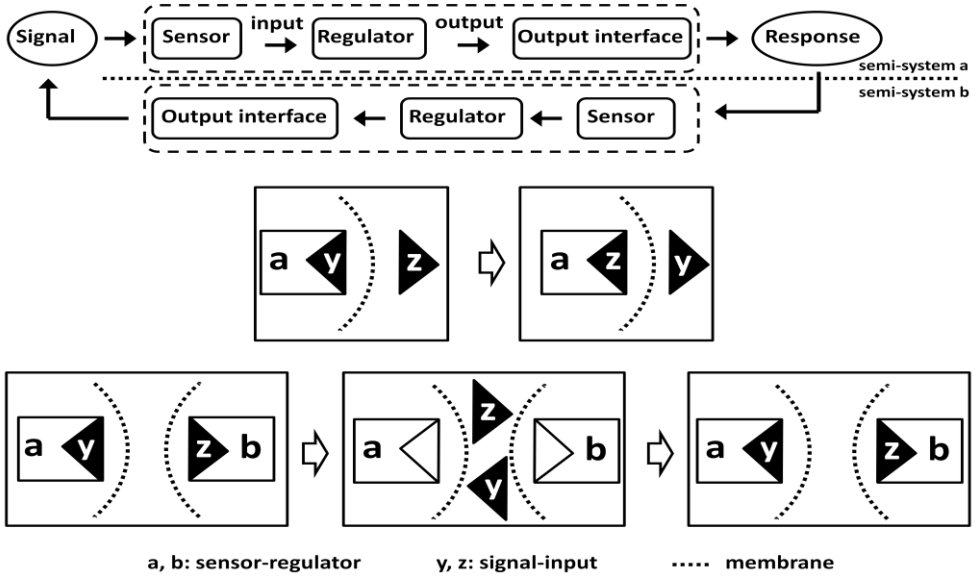


Figure 5.5 Representation of a communication system and its hydrazone-based adaptation.

The chemical networks shown in this work can be regarded as a simplified version of the communication by chemical transport that takes place in living organisms. For example, hormones that regulate functions in an organism, can cross the cell membrane and bind to specific receptors, releasing chemicals able to trigger important biological actions within the organism (Figure 5.6). Similar to the systems shown in this chapter, the concentration of these hormones is proportional to the effect that they produce when they cross the membrane. The secretion of hormones is normally regulated by a negative feedback until the proper equilibrium in the concentration is reached. In our systems, this equilibrium is defined by the minimum value of ΔG for the combined phases. There is therefore an important difference between our network and most communication processes in biology. While the former is under thermodynamic control, the latter are under kinetic control.

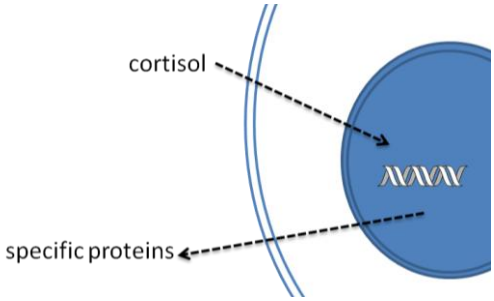


Figure 5.6 Cortisol hormone enters the cytoplasm and nucleus of a cell to bind DNA and increase the synthesis of RNA and specific proteins. The higher the cortisol concentration is, the larger is the amount of specific proteins synthesized.

5.3 Hybrid hydrazone- and disulfide-based libraries

Our group[26] and others[27] have previously developed systems that combine two different reversible exchange processes which could be alternately switched on by changing the pH value of the solution. Going one step further from the hydrazone based communication systems, we also designed a responsive chemical network featuring communication between orthogonal libraries which are simultaneously operative in two chambers at different pH values. Each of the libraries requires a specific pH range in order to be active; while hydrazone exchange needs acidic pH, disulfide libraries perform best at pH values above 7. This system prevents the mixing of both aqueous solutions maintaining the pH of each semi-system stable while allowing some specific molecules to travel through the whole system. The pH of both phases was confirmed to be stable for a period of over 2 months. The Gibbs energy of the system should decrease as molecular exchange takes place until a minimum is reached. At this point, the equilibrium of the system is reached and the composition of each of the aqueous phases remains constant.

In a similar triphasic system as previously described, semi-system 1a was kept at pH 4 containing hydrazone **BU** made by mixing hydrazide **B** and aldehyde **U** (as disulfide which was *in-situ* reduced to thiols by TCEP; see experimental part). (Figure 5.7).

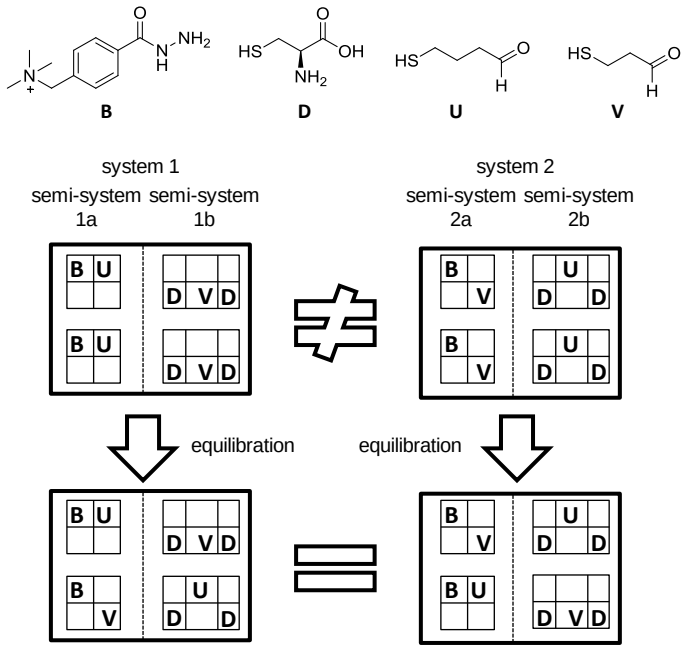


Figure 5.7 Schematic representation of the initial and final state of two initially different systems constituted by an aqueous hydrazone library and an aqueous disulfide library connected through an organic solvent.

Semi-system 1b contained disulfide **DVD** prepared by mixing thiol **D** and **V** (in disulfide form) at pH 8. The formation of both **BU** and **DVD** from their starting building blocks are equilibrium processes. **DVD** was formed by the reaction of a molecule of **V** and a molecule of **D** to build a disulfide bond and a second molecule of **D** to form a thiazolidine bond (Figure 5.8). The structure of the thiazolidine is reached via an energetically favorable five member ring. The formation of this thiazolidine ring is reversible and has been proved to be suitable for its use in dynamic combinatorial chemistry.[28]

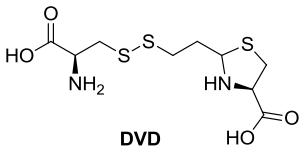
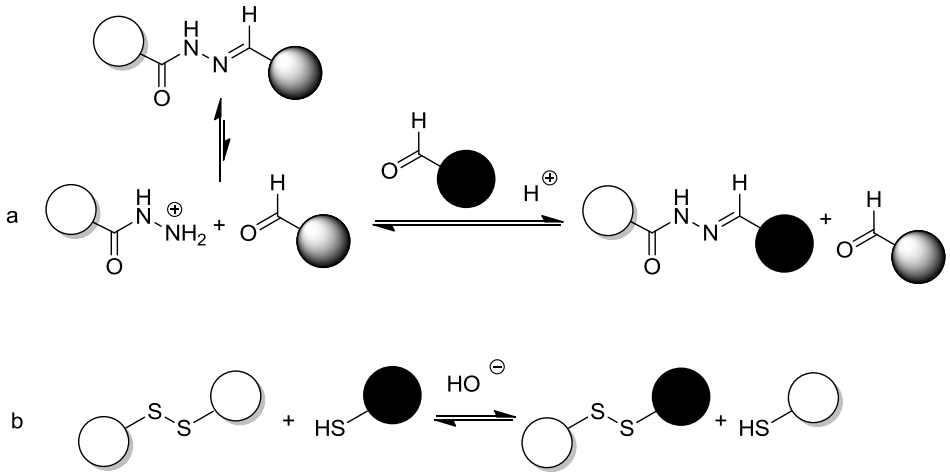


Figure 5.8 Structure of molecule **DVD**.

These two types of library members contain building blocks **U** and **V** which are able to cross the organic phase and participate in both hydrazone (Scheme 5.1a) and disulfide exchange (Scheme 5.1b).



Scheme 5.1 Hydrazone and disulfide exchange.

Molecules **U** and **V** carry an aldehyde group suitable for hydrazone exchange and a thiol group useful for building reversible disulfide bonds. The hydrazones in semi-system 1a were built from hydrazide **B** which is a positively charged species unable to migrate across the apolar organic phase. In disulfide containing semi-system 1b, cysteine was also preventing the migration of disulfides through the organic phase due to the presence of the anionic carboxylate and protonated amino groups. To diminish the formation of apolar homodimers of **U** and **V** able to escape the aqueous phase, an excess of cysteine was added to semi-system 1b, which resulted in the formation of **D** homodimers that did not disturb the communication process.

After connecting both semi-systems, the concentration of compound **BU** in system 1 decreased with time due to the transfer of mercaptoaldehyde **U** from semi-system 1a to semi-system 1b (Figure 5.9). The entrance of thiol **U** into semi-system 1b produced a re-equilibration of the system. Thiol **U** generated disulfides **DUD** by reacting with **DVD** therefore releasing mercaptoaldehyde **V** able to cross the organic barrier and reach semi-system 1a. The reaction of **V** with **BU** to form **BV** provided further release of **U** that

continued the process of re-equilibration until both semi-systems reached a stable composition after 60 days.

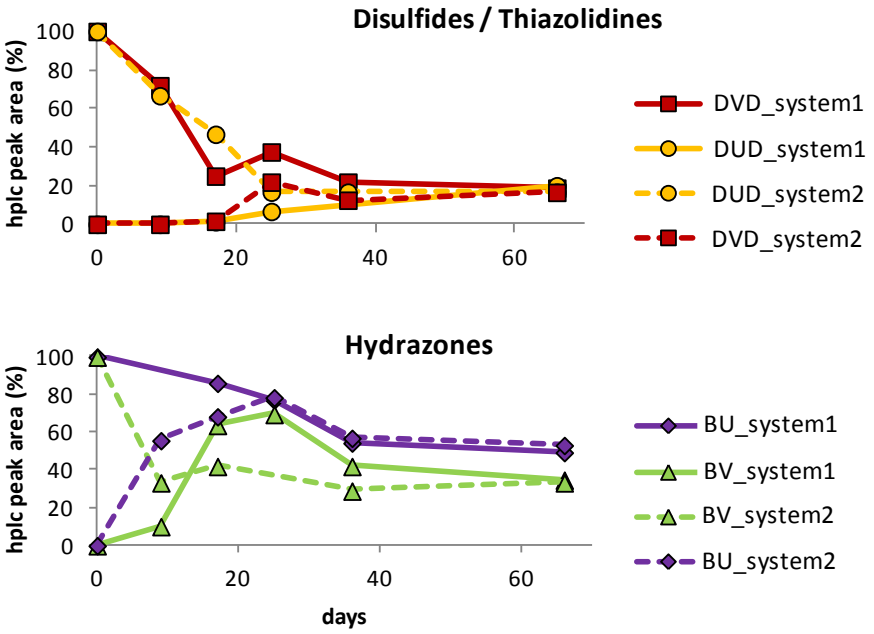


Figure 5.9 Composition shift of disulfides and thiazolidines (top) and hydrazones (bottom) expressed as percentage of total peak area of the chromatogram. The initial hydrazones and disulfides/thiazolidines in each semi-system decreased in concentration to reach a stable concentration. Similarly, the concentration of the newly formed hydrazones and disulfides rose to a stable value. The final concentrations of all the compounds in equilibrium matched well for the two systems. Conditions: See Experimental part (Chapter 5.6).

An analogous system (system 2) starting from **BV** and **DUD** was also prepared. The composition of systems 1 and 2 converged after equilibration between semi-systems (Figure 5.9). These results represent again a successful system communication, based this time on the simultaneous re-equilibration of two non-compatible (in terms of pH requirements) dynamic combinatorial libraries. The possibility of connecting combinatorial systems of such different nature enhances the prospects of dynamic combinatorial chemistry to develop complex networks under controlled conditions. For example, these newly developed systems might have application in the search of molecular binders that need different environmental conditions to the ones from the building blocks from which

they are made. Alternatively, for cascade synthetic processes it might also be interesting to consider the advantage of using these types of multiphase containers. In addition, the study of networks as the ones shown here could contribute to understand some of the mysteries of biochemical communication systems.

5.4 Communication through liposomes

In order to take a step further into mimicking biochemical systems, chemical signaling through the membrane of liposomes was tested. For that purpose, batches of liposomes were prepared using phospholipid POPC, cetrimonium bromide and cholesterol.[29]

In a first experiment, the liposomes were formed in a solution of hydrazone **AY** (Figure 5.10) in piperazine buffer 50 mM, pH 6.0 and later separated by size exclusion chromatography (SEC) from the external residual hydrazone. These liposomes were then transferred to a solution containing a different hydrazone (**BX**). The mixture was left to equilibrate for 3 days and the liposomes were separated from the external solution once again by SEC. The analysis by LC-MS of the entrapped aqueous phase of the liposomes revealed the presence of the new hydrazone **AX**.

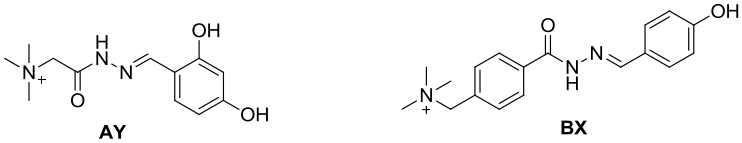


Figure 5.10 Hydrazones **AY** and **BX** used for molecular communication between the inside and the outside of liposomes.

In the external aqueous solution, the new hydrazone **BY** was also found. These findings could indicate that the presence of **AX** and **BY** is a result of the transport of aldehydes **Y** and **X** through the liposome membrane and a subsequent reequilibration. Unfortunately, also some amount of hydrazone **BX** was detected when analyzing the liposome content, as well as hydrazone **AX** in the exterior solution, indicating that the liposome membrane is permeable to these hydrazones. Due to the larger volume of external solution as compared to the entrapped volume, the presence of **X** containing hydrazones was dominant both inside and outside of the liposomes. Nevertheless, the different amount of **BX** relative to **AX** inside the liposomes allows to draw an interesting conclusion (Figure

5.11). In the entrapped solution, the concentration of **AX** was higher than that of **BX** (**AX** and **BX** have a comparable concentration/absorption signal intensity ratio at 260 nm), meaning that the transfer and exchange of aldehydes was faster than the diffusion of **BX** into the liposomes. In the hypothetic case of **BX** being the main source of **X** inside the liposomes, the amount of **BX** would always exceed (or at least equal) that of **AX**, since at equilibrium, the amount of **AX** and **BX** is comparable. The **Y** containing hydrazones had a parallel behavior to **AX** and **BX** and thus were omitted from this discussion (for mass analysis, see experimental section, Figure 5.21).

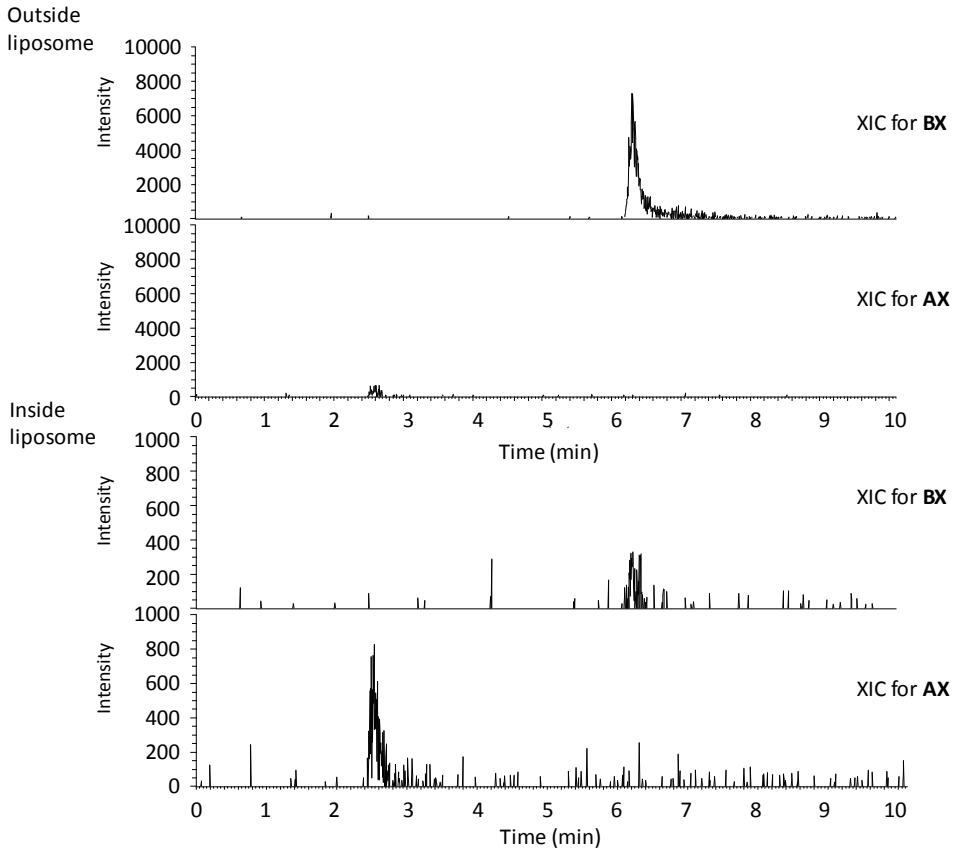


Figure 5.11 Extracted ion chromatogram (XIC) of the hydrazones **AX** (252±0.5 u) and **BX** (312±0.5 u) for the aqueous solution outside and inside of the liposomes. The liposomes were initially charged with **AY** and later exposed to a solution of **BX** (see section 5.6 for the detailed procedure). The presence of hydrazone **BX** inside the liposomes indicates that the liposome membrane is partially permeable to hydrazide/hydrazones. The presence of a greater amount of **AX** inside the liposomes implies there has been aldehyde exchange (subsequent to **A** crossing the liposomes membrane).

The solution outside the liposomes contained a higher proportion of the initial **BX** hydrazone compared to **AX** hydrazone, as expected.

In order to improve passive transport selectivity and prevent the hydrazones from passing through the membrane of the liposomes, the aldehyde residues were modified. To favor the transport of the aldehydes, aliphatic aldehydes isobutyraldehyde (**S**) and pivalaldehyde (**T**) were chosen instead of aromatic **X** and **Y** to generate hydrazones (see Figure 5.12).

AS **AT**

Figure 5.12 Hydrazones **AS** and **AT** made from hydrazide A and the corresponding aldehydes used for molecular communication between groups of liposomes.

An attempt to achieve a communication process between two families of liposomes was performed. Each family of liposomes enclosed a different hydrazone (**AS** or **AT**). Both families of liposomes were placed in an aqueous solution divided by a dialysis membrane that prevented the liposomes from mixing but allowed the free aldehydes to cross. After letting the system equilibrate for 4 days, the analysis of the content by LC-MS did not show any signals for hydrazone products. A plausible explanation for the lack of hydrazones is an exodus of the aldehydes from the inside of the liposomes to the external aqueous solution. Free hydrazide **A** was detected inside the liposomes, supporting this hypothesis.

The conditions for the communication between liposomes via aldehyde exchange could not be optimized so far. Possible improvements may be obtained by increasing the hydrazone concentration, or increasing the ratio of entrapped relative to external volume of the liposome solutions.

5.5 Conclusions

Dynamic combinatorial chemistry has been used to achieve communication between systems of aqueous dynamic combinatorial libraries connected through a bulk of organic solvent. The systems were prepared using one type of reversible chemistry (hydrazone exchange) or two orthogonal chemistries (hydrazone and disulfide exchange). Liposomes were tested as cell-like containers for analogous libraries.

The combinatorial libraries consisted of hydrazones able to release aldehydes that behaved as signals travelling through the organic layer to interact with the neighboring aqueous compartment. The hydrazones chemically perceived these signals and reacted to them by shifting their equilibrium and releasing new signals until both compartments reached a new equilibrium state which depends on the equilibrium constants of the hydrazones involved in the system. Hybrid systems containing separate hydrazone and disulfide libraries at different pH values were allowed to communicate through mercaptoaldehydes as signaling molecules. These bifunctional species participated in the equilibration of dynamic combinatorial libraries through thiol and aldehyde moieties when present in the disulfide compartment and through an aldehyde group when in the hydrazone compartment.

The results here presented are the first examples of designed three-phase systems as a new device to interface networks that require incompatible conditions or chemical species and which can be in thermodynamic equilibrium by contact through a non-miscible phase, increasing the versatility of dynamic combinatorial chemical systems. Finally, these innovative systems represent a simplified example of intersystem communication, mimicking signaling across cell membranes. Therefore a potential use in the study and understanding of complex communication behavior such as quorum sensing is anticipated.

5.6 Experimental

Reagents and solvents

All reagents and solvents were obtained from commercial sources and used without further purification unless specified otherwise.

NMR analysis

NMR spectra were obtained on a Varian AS 400 MHz instrument or Varian AC 300 MHz. ^{1}H chemical shifts are reported as δ in ppm relative to residual protonated solvent resonances. ^{13}C chemical shifts are reported as δ in ppm and measured relative to solvent references.

HPLC analysis

Analytic HPLC was carried out on Hewlett Packard 1050 or 1100 systems coupled to UV detectors and the data were processed using HP Chemstation software. Separations were performed on a reversed phase Waters symmetry C8 column (4.6 x 150 mm, 3.5 µm particle size). Aliquots of 3 µL of library solution were injected. Doubly distilled water, HPLC-S-grade acetonitrile from Biosolve and formic acid were used to prepare the eluents:
eluent A = water + 5.0 % acetonitrile + 0.10 % formic acid
eluent B = acetonitrile + 5.0 % water + 0.10 % formic acid
Chromatography was performed at 45 °C using UV detection at 260 nm and a constant flow rate of 1.00 mL/min. The HPLC analysis method was as follows:

Time (min)	Eluent A (%)	Eluent B (%)
0	100	0
5	89	11
16	33	66
22	0	100
26	0	100
27	100	0
34	100	0

System design

The connection of the two aqueous phases was made via an organic solvent. Its function is to prevent the mixing of the two different aqueous phases, and to limit the exchange of some chemical species while allowing some others to cross. After testing some solvents (hexane, heptanes, CH_3Cl), EtOAc was chosen since it afforded sufficient solubility of the aldehydes (as opposed to hexane or heptanes) while avoiding solubilization of the charged hydrazides and hydrazones (as observed for chlorinated solvents).

The container with which the communication experiments were performed was an H-shaped glass tube (Figure 5.13). Each of the phases contained 7.0 mL of solvent. For the experiment with only hydrazones, the concentration of the hydrazones was 7.50 mM in ammonium formate buffer 100 mM, pH 4.0 (adjusted with formic acid). An additional 2.50 mM of hydrazide was added in order to accelerate the hydrazone exchange.

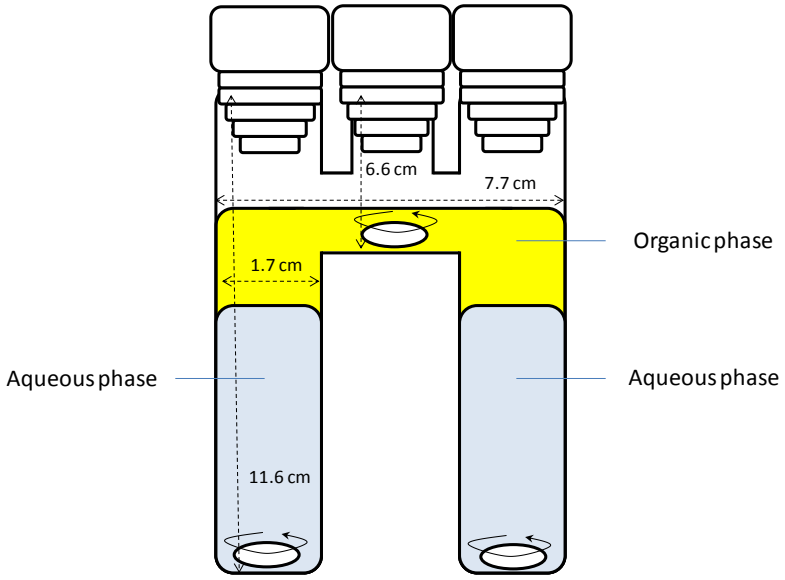


Figure 5.13 Schematic representation of the container used for the connection of libraries. The volume of each phase was 7.0 mL.

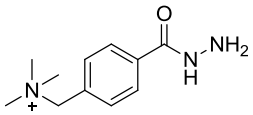
To set up the system with a combination of hydrazones and disulfides, hydrazone **BU** was prepared in semi-system 1a by mixing dimer of mercaptoaldehyde **U** (5.00 mM) with **B** (10.0 mM) in ammonium formate buffer 100 mM pH 4.0 (7.0 mL) in presence of TCEP

(20.0 mM) to reduce disulfides into thiols. The same approach was used to prepare **BV**. **DVD** and **DUD** were prepared by mixing cysteine (40.0 mM) and **U** or **V** (5.00 mM) in borate buffer 50 mM pH 8.0 (7.0 mL).

Synthetic procedures

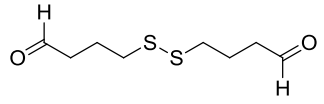
The synthesis of the hydrazones from the corresponding hydrazides and aldehydes was performed by dissolving both reagents in buffer and allowing for full conversion (typically overnight). Whenever isolation of the hydrazone was required, both hydrazone and aldehyde were dissolved in CH_2Cl_2/DMSO (50/50), formic acid was added as catalyst and the solution was stirred overnight at r.t. Afterwards, the product was precipitated by adding EtOAc to the solution. The hydrazone was then filtered out and dried.

1-(4-(Hydrazinecarbonyl)phenyl)-N,N,N-trimethylmethanaminium (**B**)



Methyl 4-bromomethylbenzoate (2.60 g, 11.0 mmol) was refluxed with trimethylamine (3.5 mL 45% aq) in 20 mL of EtOH for 4 hours and the solvents were then evaporated under reduced pressure. The resulting mixture was dissolved in 20 mL of H_2O and 3 mL of aqueous hydrazine (60% conc) was added and refluxed for 6 hours. The solvents were removed under reduced pressure. The crude product was washed by sonicating in EtOH, then filtered out and dried under vacuum. The final product was obtained as a white powder (3.02 g, 99% yield). ^{1}H-NMR (400 MHz, DMSO/D_2O 1/1): δ 9.90 (s, 1H), 7.93 (d, *J* = 8.9Hz, 2H), 7.60 (d, *J* = 8.9Hz, 2H), 4.57 (s, 2H), 3.02 (s, 9H). ^{13}C-NMR (100 MHz, DMSO/D_2O 1/1): δ 165.7 (O=C(NH-)-), 132.4 (C_{quat}), 131.3 (2xCH), 129.0 (C_{quat}) 125.8 (2xCH), 66.5 (CH_2), 50.5 (3xCH_3). HR-MS calcd (M-H^+) 208.1444, found 208.1435.

4-Mercaptobutanal (**W**) (as disulfide dimer)

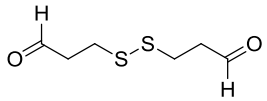


Ethyl 4-bromobutanoate (27.3 g, 140 mmol) was refluxed in EtOH (125 mL) together with thiourea (16.0 g, 210 mmol) for 3h. NaOH (10.0 g, 250 mmol) was added and the mixture was further refluxed for 2 more hours. HCl conc (25 mL) was added and all the solvents were removed under vacuum. The remaining oil was redissolved in EtOH (150

mL) and I_2 pellets were gradually added. The solution turned initially red due to the presence of I_2 but it readily become pale as the I_2 was reduced to I^- by the thiols in solution which became oxidized to disulfides. Iodine pellets were added until the solution remained red for more than 30 minutes. The solution was stirred overnight. The EtOH was removed *in vacuo* and the product was purified by column chromatography in EtOAc/Heptane (10/1, 3/1, 1/1). An overall yield of 14% (2.95 g) of diethyl 4,4'-disulfanediyldibutanoate was obtained. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 4.10 (q, *J* = 8.0Hz, 4H), 2.69 (t, *J* = 7.2Hz, 4H), 2.40 (m, 4H), 2.01 (m, 4H), 1.23 (t, *J* = 7.6Hz, 6H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 172.8 (2xO=C(O)-), 60.4 (2xCH_2), 37.8 (2xCH_2), 32.6 (2xCH_2), 24.2 (2xCH_2), 14.2 (2xCH_3). HR-MS calcd (M-H^+) 295.1038, found 295.1042.

To reduce the carboxylate ester groups to aldehyde groups, 2.80 g (9.70 mmol) of the product from the last synthetic step were dissolved in dry CH_2Cl_2 (50 mL) under a N_2 atmosphere and the mixture was cooled to -78 °C. A 1.0 M solution of DIBAL-H in CH_2Cl_2 (19.6 mL, 19.6 mmol) was added dropwise while stirring for 1h.[30] The reaction was stirred for 1 more hour and then it was quenched with 20 mL of aqueous HCl 10%. The product was extracted with CH_2Cl_2. The solution was dried over Mg_2SO_4 and the solvent removed under vacuum, giving 1.23 g (70% yield) of product. Rf = 0.59 in AcOEt/Hept (1/1). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 9.73 (s, 2H), 2.65 (t, 8.0Hz, 4H), 2.54 (t, 8.0Hz, 4H), 1.96 (q, 8.0Hz, 4H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 201.4 (2xO=C(H)-), 42.1 (2xCH_2), 37.6 (2xCH_2), 21.3 (2xCH_2). HR-MS calcd (M-H^+) 207.0508, found 207.0511.

3-Mercaptopropanal (**X**) (as disulfide dimer)



This compound was prepared following a modified procedure described in the literature:[31]

Methyl 3-mercaptopropionate (5.00 g, 41.7 mmol) was added to 100 mL MeOH. I_2 pellets were gradually added. The solution turned initially red due to the presence of I_2 but it readily became pale as the I_2 was reduced to I^- by the thiols in solution which became oxidized to disulfides. Iodine pellets were added until the solution remained red for more than 30 minutes. The solution was stirred overnight. The solvent was removed *in vacuo* and the product was purified by column chromatography using EtOAc/Heptane (1/1) (R_f = 0.68). Dimethyl 3,3'-disulfanediyldipropanoate (2.34 g, 48%

yield) was obtained and used for the next synthetic step. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.70 (s, 6H), 2.92 (t, *J* = 10Hz, 4H), 2.74 (t, *J* = 10Hz, 4H). See reference[31] for analytical data.

The product mentioned above (2.34 g, 9.80 mmol) was dissolved in dry CH_2Cl_2 (50 mL) under a N_2 atmosphere and the mixture was cooled to -78 °C. A 1.0 M solution of DIBAL-H in CH_2Cl_2 (19.6 mL, 19.6 mmol) was added dropwise while stirring for 1h. The reaction was stirred for 1 more hour and then quenched with 20 mL of aqueous HCl 10%. After extraction with CH_2Cl_2, 0.82 g (47% yield) of product was obtained. Rf = 0.55 in AcOEt/Hept (1/1). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 9.82 (s, 2H), 2.92 (m, 8H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 199.8 (2xO=C(H)-), 43.1 (2xCH_2), 30.0 (2xCH_2). HR-MS calcd ($M\text{-}H^+$) 178.0122, found 178.0117.

Preparation of liposomes

POPC was bought from Genzyme, cholesterol from Avanti lipids and cetrimonium bromide from Aldrich.

Liposome formulation for 3.0 mL:

POPC (MW 760.076) 11.4 mg (final concentration 5.00 mM), cetrimonium bromide (MW 364.45) 0.30 mg (final concentration 0.25 mM) and cholesterol (MW 386.65) 0.30 mg (final concentration 0.25 mM) were mixed together in 3.0 mL of $CHCl_3$ until complete dissolution. The organic solvent was removed under a nitrogen flow, forming a lipid film. The lipid film was dried overnight under reduced pressure and then hydrated with 3.0 mL of a selected hydrazone solution, vortexed for 10 seconds and incubated for 5 minutes. Then the solution was subjected to 20 cycles of freeze-thaw (liquid nitrogen; 30 °C water). The solution was again incubated for 5 minutes and then passed through a Sephadex NAP-25 column (Illustra, GE Healthcare) equilibrated and eluted with the buffer used for the experiment.

For the experiment involving a single type of liposomes containing one hydrazone, the solution of the external hydrazone was added to the liposome solution and left to equilibrate for 7 days.

For the experiment involving two types of liposomes containing a different hydrazone each, the two liposome solutions were separated with a Spectra/pore 7 (spectrumlabs.com) dialysis membrane (MWCO 1000) previously hydrated with the buffer used for the experiment, and the mixture of liposomes was left to equilibrate for 7 days.

LC-MS analysis

For the LC-MS measurements an Accela High Speed LC system (ThermoFisher Scientific, Courtaboeuf, France) was coupled to a LTQ-Fleet Ion Trap Mass Spectrometer. Mass spectra (positive ion mode) were obtained using the following conditions: sheath gas flow rate 30, aux. gas flow rate 10, sweep gas flow rate 5, ionization spray voltage 3.50 kV, capillary temperature 330 °C, capillary voltage 12 V, tube lens 40 V.

The LC method employed was the same as that one used for HPLC. The flow was split after the LC to allow 0.30 mL/min to enter the mass spectrometer.

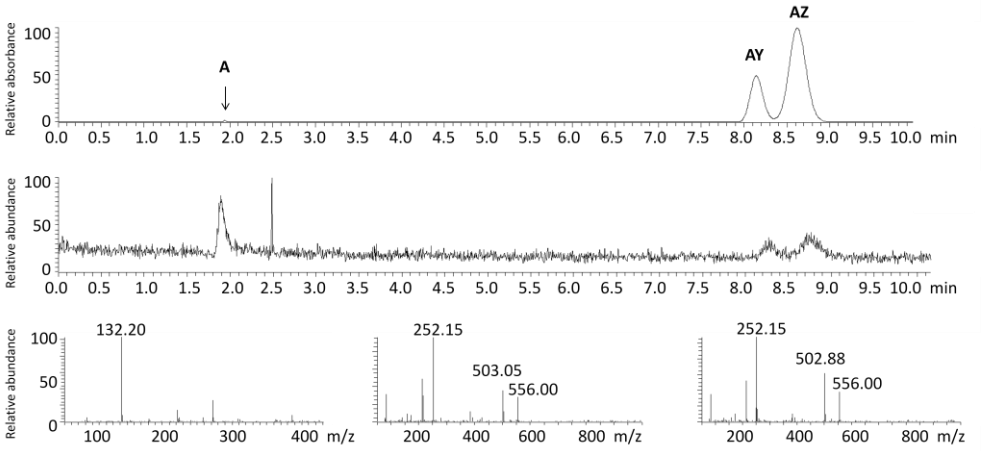


Figure 5.14 LC-MS analysis of in semi-system 2a initially containing only hydrazone **AZ** after 10 days of equilibration. HPLC trace (top), TIC (middle) and mass spectra (bottom) of the three main peaks in the TIC corresponding to **A** (bottom, left; $M+H^+$ calcd m/z= 132.11, obs m/z= 132.20) **AY** (bottom, middle; $M+H^+$ calcd m/z= 252.13, obs m/z= 252.15) and **AZ** (bottom, right; $M+H^+$ calcd m/z= 252.13, obs m/z= 252.15). TFA was used instead of F.A. in this analysis.

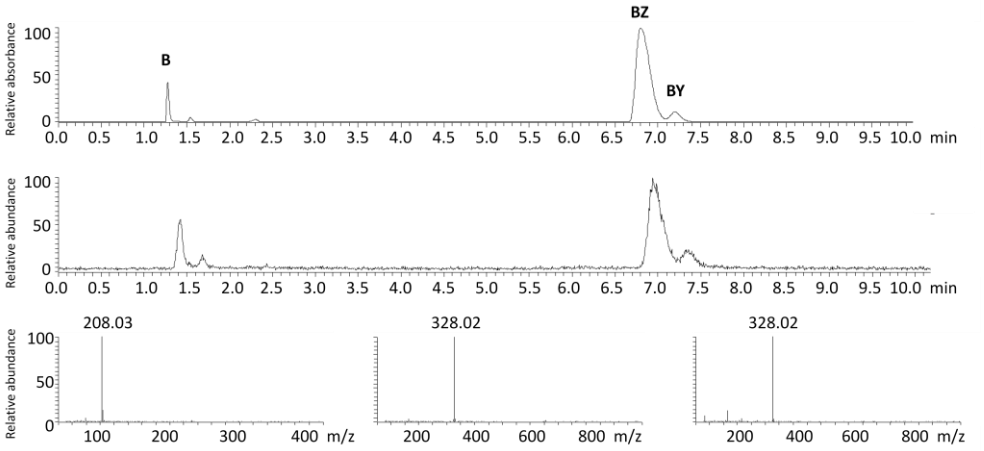


Figure 5.15 LC-MS analysis of in semi-system 2b initially containing only hydrazone **BY** after 10 days of equilibration. HPLC trace (top), TIC (middle) and mass spectra (bottom) of the three main peaks in the TIC corresponding to **B** (bottom, left; $M+H^+$ calcd m/z= 208.14, obs m/z= 208.03) **BZ** (bottom, middle; $M+H^+$ calcd m/z= 328.17, obs m/z= 328.02) and **BY** (bottom, right; $M+H^+$ calcd m/z= 328.17, obs m/z= 328.02).

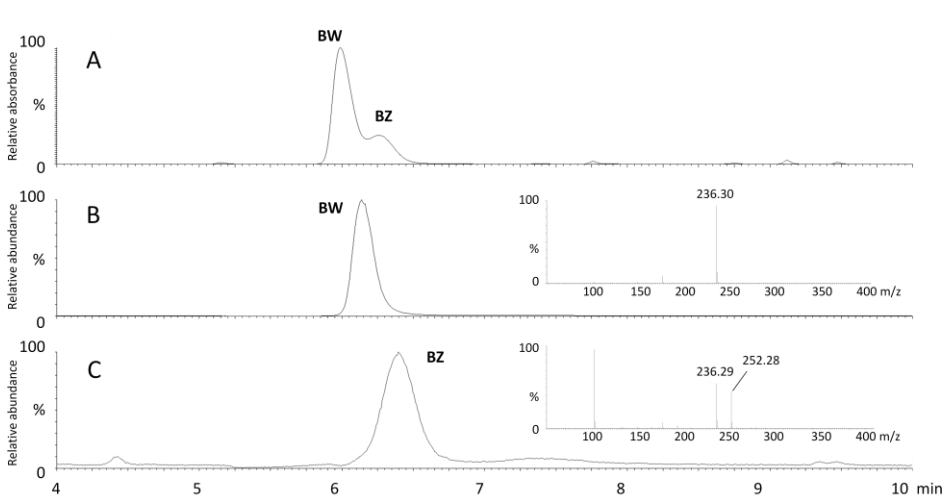


Figure 5.16 HPLC trace of system 1a where both **BW** and **BZ** are present (A). XIC (extracted ion chromatogram) mass for **BW** (m/z= 235.5-236.5) and mass spectrum ($M+H^+$ calcd m/z= 236.14, obs m/z= 236.30) (B). XIC for **BZ** (m/z= 251.6-252.6) and mass spectrum ($M+H^+$ calcd m/z= 252.13, obs m/z= 252.28) (C) (residual **BW** can be observed).

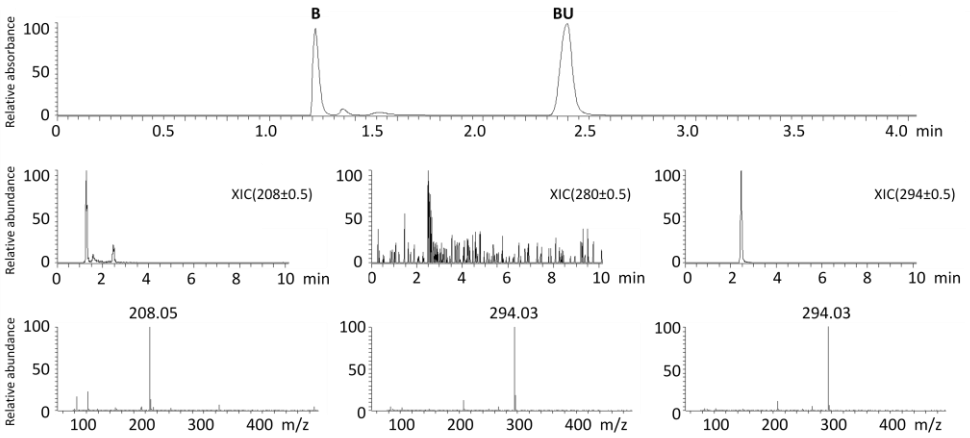


Figure 5.17 HPLC trace of semi-system 1a of the mixed hydrazone-disulfide libraries at time=0 (top) and XIC (extracted ion chromatogram) for **B** (middle, left), **BV** (middle, center) and **BU** (middle, right) and the mass spectra of the main peaks of the respective XICs ($M+H^+$ calcd m/z= 208.14, obs m/z= 208.05; $M+H^+$ calcd m/z= 280.15, obs m/z= ---; $M+H^+$ calcd m/z= 294.16, obs m/z= 294.03) (bottom). At this stage, no **BV** was detectable.

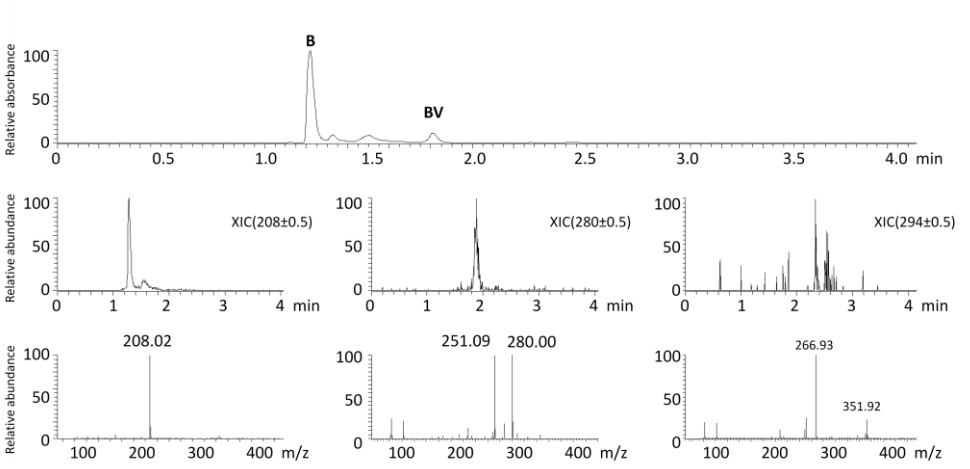


Figure 5.18 HPLC trace of semi-system 2a of the mixed hydrazone-disulfide libraries at time=0 (top) and XIC (extracted ion chromatogram) for **B** (middle, left), **BV** (middle, center) and **BU** (middle, right) and the mass spectra of the main peaks of the respective XICs ($M+H^+$ calcd m/z= 208.14, obs m/z= 208.02; $M+H^+$ calcd m/z= 280.15, obs m/z= 280.00; $M+H^+$ calcd m/z= 294.16, obs m/z= ---) (bottom). At this stage, no **BU** was present.

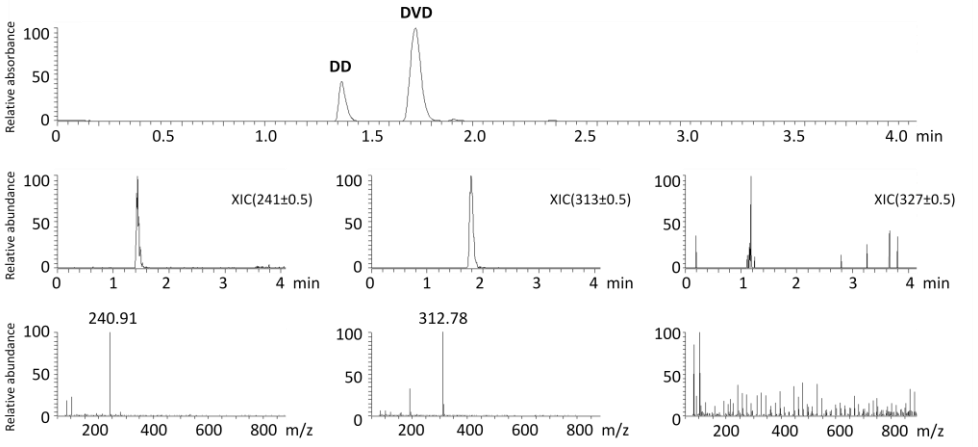


Figure 5.19 HPLC trace of semi-system 1b of the mixed hydrazone-disulfide libraries at time=0 (top) and XIC (extracted ion chromatogram) for **DD** (middle, left), **DVD** (middle, center) and **DUD** (middle, right) and the mass spectra of the main peaks of the respective XICs ($M+H^+$ calcd m/z= 241.03, obs m/z= 240.91; $M+H^+$ calcd m/z= 313.03, obs m/z= 312.78; $M+H^+$ calcd m/z= 327.05, obs m/z= ---) (bottom). At this stage, no **DUD** was present.

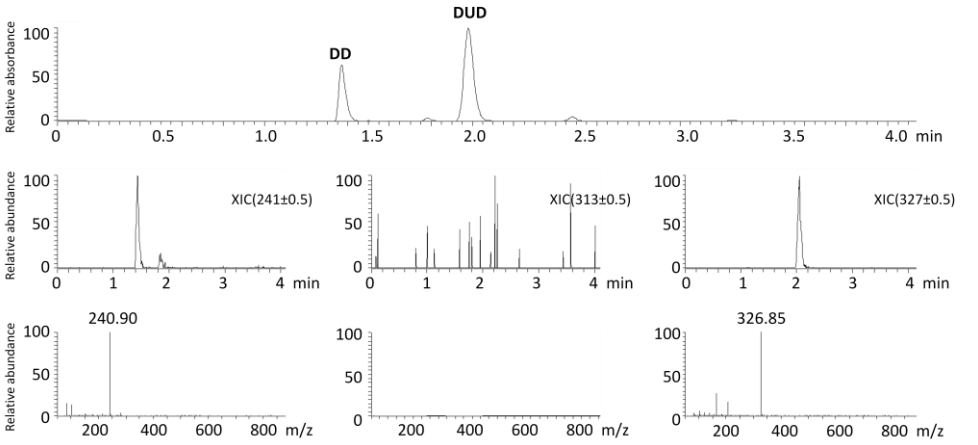


Figure 5.20 HPLC trace of semi-system 2b of the mixed hydrazone-disulfide libraries at time=0 (top) and XIC (extracted ion chromatogram) for **DD** (middle, left), **DVD** (middle, center) and **DUD** (middle, right) and the mass spectra of the main peaks of the respective XICs ($M+H^+$ calcd m/z= 241.03, obs m/z= 240.90; $M+H^+$ calcd m/z= 313.03, obs m/z= ---; $M+H^+$ calcd m/z= 327.05, obs m/z= 326.85) (bottom). At this stage, no **DVD** was present.

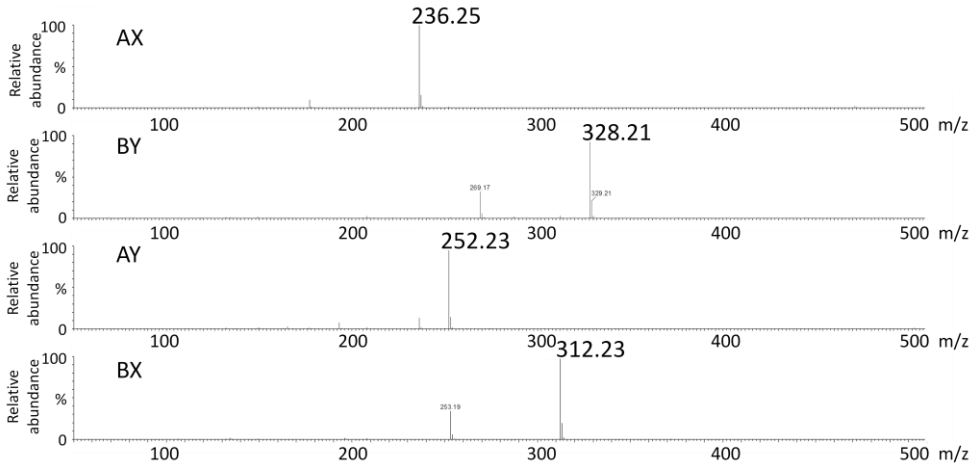


Figure 5.21 Mass spectra of the **A**- and **B**-derivatives used for chemical communication between internal and external part of liposomes: **AX** ($M+H^+$ calcd m/z= 236.14, obs m/z= 236.25), **BY** ($M+H^+$ calcd m/z= 328.17, obs m/z= 328.21), **AY** ($M+H^+$ calcd m/z= 252.13, obs m/z= 252.23) and **BX** ($M+H^+$ calcd m/z= 312.17, obs m/z= 312.23).

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Chapter 6

Conclusions and Perspectives

6.1 Introduction

Dynamic combinatorial chemistry appears to be gradually finding its niche within chemistry. As more investigations on the basics and uses of DCC are carried out, an increasing attention is being drawn towards it. As a relatively young field of research, some foundations needed to be initially set. After an initial period of three decades of discovering new possibilities, the time has arrived to start profiting from the possibilities offered by DCC. Even though promising results have been achieved, mainly only proof-of-principle examples have been reported. The lack of general and effective applications has prevented DCC from becoming a main discussion topic among chemists. Up to now, the major uses of DCC are related to the discovery of receptors. However, the recent applications of DCLs to catalysis should hopefully serve as a motivation for researchers to consider DCC as a real alternative in the discovery of new catalytic approaches, contributing to the development of DCC. Much of the work described in this thesis aimed to explore the possibilities that disulfide and hydrazone dynamic combinatorial libraries offer for the identification and development of catalysts (Figure 6.1). Taking advantage from the ease of DCLs to yield complex molecular networks, part of the work described in this thesis was dedicated to the development of dynamic molecular arrays. Their reequilibration was promoted through a membrane, in an attempt to extend the functionality of DCC.

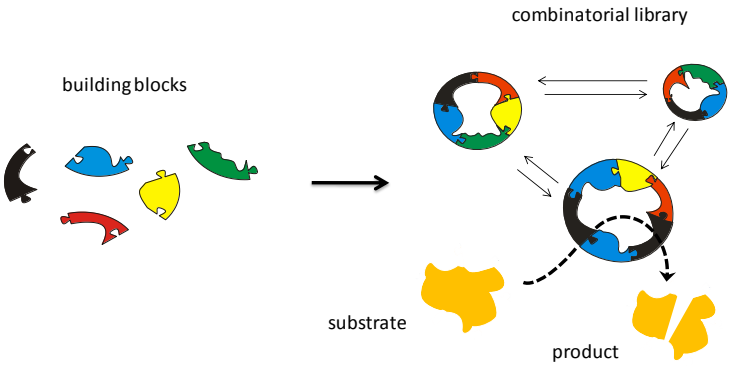


Figure 6.1 Schematic representation of a catalytic dynamic combinatorial library

6.2 Research overview

The suitability for catalytic purposes of two types of reversible covalent chemistry, disulfides and hydrazones, was tested by means of the preparation and study of dynamic combinatorial libraries in absence and presence of metals.

Cyclen was chosen as the metal binding unit included in the building blocks which participated in the formation of the DCLs. The presence of Zn(II) in the hydrazide building block proved to be a valid catalytic center for the hydrolysis of 4-NPA. The hydrazone libraries made from the same building block also showed catalytic activity (Chapter 2). These positive results can be considered as promising and set a precedent for the development of self-assembling catalytic libraries.

In Chapter 3, the results of the research devoted to the testing of disulfide dynamic combinatorial libraries were shown. Libraries containing combinations of dithiol building blocks were obtained, proving cyclen a compatible function in disulfide libraries. The presence of metals, however, disturbed the stability of the libraries by causing precipitation problems and alterations in the oxidation rate of the libraries when compared to the ones in absence of metals.

By using naphthalene-based dithiol building blocks, catalytic libraries able to react to the presence of a substrate were formed (Chapter 4). The presence of an aza-Cope rearrangement substrate in a combinatorial library induced the shift of the library composition to form the catalytic species that accelerated the rearrangement reaction. After the substrate reacted away, the library changed back to its initial composition. The libraries had been previously screened with TSAs to identify stabilizing interactions that could lead to a decrease in energy of the TS of the reaction. The work presented in this chapter yielded the first successful responsive catalytic libraries.

In Chapter 5, systems containing separated DCLs able to interact with each other were studied. Hydrazone libraries in aqueous solvent were connected through an organic bulk allowing the transfer of certain apolar molecules that acted as signals. The composition of each of the connected libraries varied depending on the distribution of the library members of the opposite library. An analogous system of communicating hydrazone and disulfide exchange reactions was also developed. For the first time, the simultaneous equilibration of these two types of DCLs was obtained. Moreover, preliminary results in

the communication of molecular networks enclosed in liposomes were also obtained. The development of these chemical communication systems might lead to their use as a simplified version of biological communication structures.

6.3 Research perspectives

Advance in the development of catalysis within DCC has been certainly made during the research reported throughout this thesis. However, further work is needed in order to solve fundamental issues frustrating the establishment of a general methodology to reveal new catalysts. Difficulties in this regard, may include the rational prediction of TSA structures which effectively imitate the real TS of the reaction aimed to be catalyzed (Chapter 4). By now, the collection of successful examples, probably in combination with molecular modeling, seems to be the only possible way to achieve a sufficient knowledge for the prediction of valid TSAs.

Other opportunities for improvement may come from the solubility problems experienced with cyclen building blocks (Chapter 2 and Chapter 3) which could be avoided by using multiphase combinatorial systems as the ones presented in the Chapter 5. Furthermore, potential incompatibilities between the substrate and the library conditions in any catalytic DCL could be a matter of concern which might be solved by a proper application of the mentioned triphasic systems.

An important inconvenience observed in the DCLs involving the cyclen moiety was the lack of templating effects. The composition of the library was not affected by the presence of any external molecule. This fact implies that the presence of possible catalysts will not be detected by simple analysis of the library after addition of the substrate or the TSA, but rather a kinetic study of the catalytic reaction is needed in presence of the DCL. After proving that certain substrates were able to bind to the individual building blocks containing cyclen, a plausible explanation to the absence of a shift in composition of the libraries is that the binding of the potential templates took effect without interacting with the rest of the structure of the library members. The location of the face of the cyclen holding the metal ion towards the outside of the building block would keep the guest away from the library member, therefore rendering the potential template inactive. In this situation, the substitution of the building block containing the metal ion by a more rigid

structure that ensures the proximity of the bound guest to the rest of the library member would increase the possibility of detecting catalytic effects within the DCLs.

The accomplishment of DCLs with catalytic behavior is important for the generation of molecular feedback systems mimicking biological control systems. When a DCL produces supramolecular structures able to interact with the products of the catalyzed reaction, feedback processes may emerge, as the equilibrium state of the library will change due to that host-guest interaction. As shown in Figure 6.2, the binding of library members to the reaction product will drive the library equilibrium to the formation of more of the binder. When this library member is formed by building blocks which are part of the catalyst, the increase of the reaction product concentration will induce a decrease in catalyst concentration, resulting in the deceleration of the reaction. On the contrary, the increase of concentration of a receptor which does not contain building blocks forming the catalyst, will free catalytic building blocks that will be able to self assemble into more catalyst, increasing the rate of the catalyzed reaction.

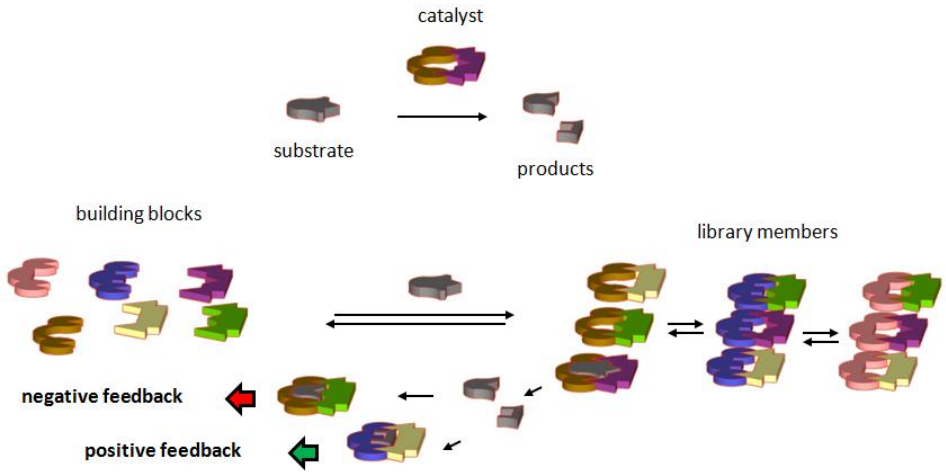


Figure 6.2 Representation of a dynamic combinatorial library of which one of the members acts as the catalyst of a chemical reaction. The binding of the reaction products to any library member containing brown or purple building blocks would induce a negative feedback, as the library would amplify this member at the expense of the catalyst. The binding of the reaction products to any other library member would favor the production of more of the catalytic library member.

Multi-phase systems of DCLs, as the ones presented in Chapter 5, might be beneficial for the generation of the mentioned feedback systems, as well as for merely synthetic purposes via selective cascade processes. For example, a substrate could go through a series of specific transformations in different phases influenced by the equilibrium state of the DCL.

In conclusion, new dynamic combinatorial libraries have been implemented during the course of this thesis work. The catalytic potential of some of the libraries and the multi-phase combinatorial systems discovered, may constitute the foundations of promising complex systems with emergent properties where the behavior of the whole network cannot be explained by only the effect of the individual components.

Summary

The gap between chemistry and biology, though apparently modest, seems to be in practice difficult to bridge. The multiple and not fully known molecular interactions between macromolecules is the first difficulty that chemists face when trying to approach the behavior of biological systems, leaving this interfacial area of science relatively unexplored and underexploited.

Dynamic Combinatorial Chemistry (DCC) is a relatively new chemical tool that allows the synthesis of complex molecules from simpler molecular building blocks able to react among themselves in a reversible way (Figure 1). The interactions of a Dynamic Combinatorial Library (DCL) of molecules with specific targets leads to composition changes of the library which can reveal potential guests and / or catalysts.

In this thesis some chemical systems have been proposed to achieve a certain level of molecular complexity and interactions within molecular networks by using DCC. These systems were implemented for the discovery of catalysts and the construction of molecular communication networks.

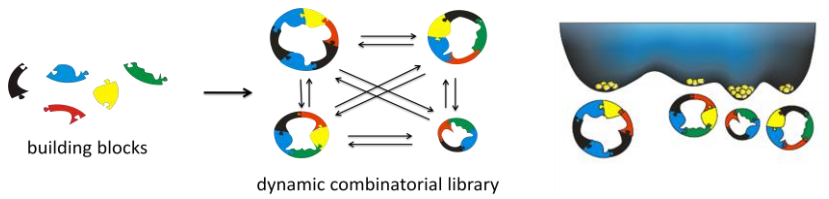


Figure 1 A dynamic combinatorial library made from different combinations of building blocks. The library members are constantly exchanging building blocks. The final composition of the library will depend on the stability of each library member.

Chapter 1 displays an overview of the main terms and definitions related to dynamic combinatorial chemistry alongside with its usage and representative examples. An introduction to catalysis in supramolecular chemistry, a main topic in this thesis, is also found in the first chapter.

In Chapter 2 the suitability of reversible hydrazone chemistry for catalysis was tested using dynamic combinatorial libraries in absence and presence of metals. Cyclen was chosen as

a metal binder unit which was included in the building blocks participating in the formation of the DCLs (Figure 2). The presence of Zn(II) in the hydrazide building block proved to be a valid catalytic center for the hydrolysis of 4-NPA. The hydrazone libraries made from the same building block also showed catalytic activity. These positive results set a precedent for the development of self-assembling catalytic libraries.

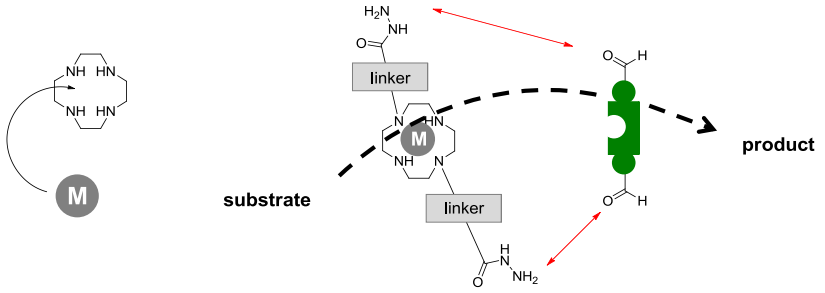


Figure 2 The goal: a metal-cyclen building block reacting with dialdehyde compound to form a hydrazone compound able to perform catalysis.

In Chapter 3 the results of the research devoted to the testing of disulfide dynamic combinatorial libraries were shown. Libraries containing combinations of dithiol building blocks were obtained, proving cyclen a compatible function in disulfide libraries. The presence of metals, however, disturbed the stability of the libraries by causing precipitation problems and alterations in the oxidation rate of the libraries when compared to the ones in absence of metals.

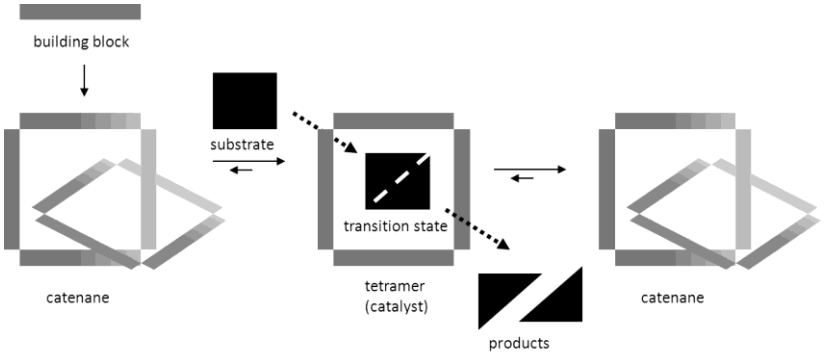


Figure 3 A solution of building blocks gives rise to a dynamic combinatorial library where the dominant library member changes from the initial catenane to the catalytic tetramer upon addition of the reaction substrate. Once the substrate is consumed, the DCL shifts back towards the initial catenanes.

The results of dithiol catalytic libraries able to react to the presence of a substrate are presented in Chapter 4. The introduction of an aza-Cope rearrangement substrate in a dynamic combinatorial library, induced the shift of the library composition to form the catalytic species that accelerated the rearrangement reaction. After the substrate reacted away, the library changed back into its initial composition (Figure 3). The libraries had been previously screened with TSAs to identify stabilizing interactions that could lead to a decrease in energy of the TS of the reaction. The work presented in this chapter yielded the first successful responsive catalytic libraries.

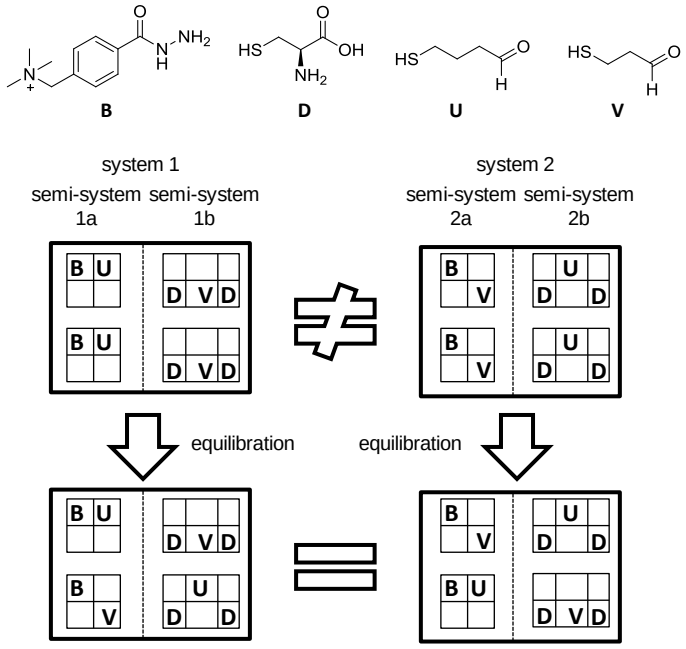


Figure 4 Schematic representation of the initial and final state of two initially different systems constituted by an aqueous hydrazone library and an aqueous disulfide library connected through an organic solvent.

In Chapter 5, systems containing separated DCLs able to interact with each other were studied. Hydrazone libraries in aqueous solvent were connected through an organic bulk allowing the transfer of certain apolar molecules that acted as signals. The composition of each of the connected libraries varied depending on the distribution of the library members of the opposite library. An analog system communicating hydrazone and disulfide exchange reactions was also developed (Figure 4). For the first time, the

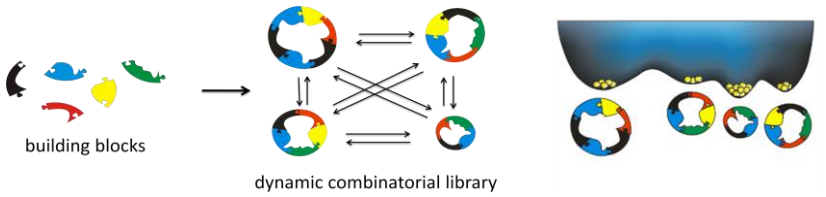
simultaneous equilibration of these two types of DCLs was obtained. Moreover, preliminary results in the communication of molecular networks enclosed in liposomes were also obtained. The development of these chemical communication systems might lead to their use as a simplified version of biological communication structures.

Samenvatting

Op het eerste oog is de kloof tussen scheikunde en biologie smal, maar in de praktijk valt dit vaak toch tegen. Het eerste probleem waar chemici tegenaan lopen zijn de vele niet volledig begrepen moleculaire interacties tussen macromoleculen en hun gedrag in biologische systemen. Dit leidt tot een grensgebied van wetenschap die relatief weinig benut wordt en verkend is.

Dynamic combinatorial chemistry (DCC) is een relatief nieuwe chemisch hulpmiddel die het mogelijk maakt om eenvoudigere moleculaire building blocks onderling en reversibel te laten reageren tot complexe moleculen (Figuur 1). De interacties tussen moleculen via de Dynamic Combinatorial Library met specifieke targetmoleculen leiden tot compositie veranderingen van de Library, waardoor potentiële guests en/of katalysatoren kunnen worden onthuld.

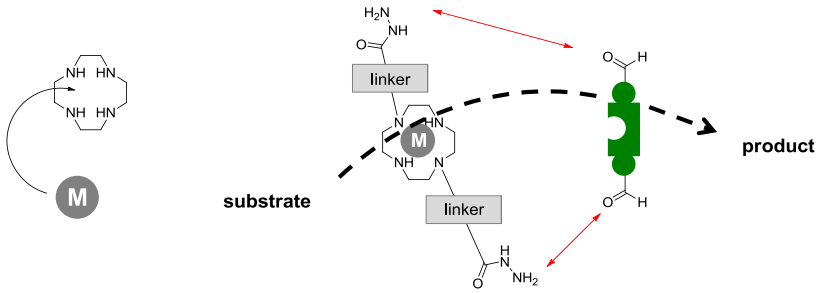
In deze thesis worden, met behulp van DCC, enkele chemische systemen voorgesteld waardoor een zeker niveau van moleculaire complexheid en interacties wordt bereikt binnen de moleculaire netwerken. Deze systemen werden ingezet voor de ontdekking van katalysatoren en de constructie van moleculaire communicatie netwerken.



Figuur 1 Een dynamic combinatorial library gemaakt uit verschillende combinaties van building blocks. De deelnemers van de library wisselen constant van positie. De eind compositie van de library hangt af van de stabliliteit van elke individuele library deelnemer.

Hoofdstuk 1 geeft een overzicht van de gebruikte termen en definities gerelateerd aan dynamic combinatorial chemistry, naast het gebruik ervan met behulp van representatieve voorbeelden. Daarnaast ook een introductie in katalyse bij supramoleculaire chemie, het hoofdonderwerp van deze thesis.

In Hoofdstuk 2 werd de bruikbaarheid van reversibele hydrazone chemie voor katalyse getest met behulp van dynamic combinatorial libraries in zowel aanwezigheid als afwezigheid van metalen. Cyclen werd gekozen als metaal binder unit, deze werd toegevoegd bij het building block waarbij deze deelnam in de formatie van de DCL (Figuur 2). Er werd aangetoond dat de aanwezigheid van Zn(II) in het hydrazide building block een geschikt katalytisch centrum vormt voor de hydrolyse van 4-NPA. De hydrazone bibliotheek gemaakt uit hetzelfde building block vertoonde ook katalytische activiteit. Deze positieve resultaten zetten een voorbeeld voor de ontwikkeling van self-assembling katalytische libraries.

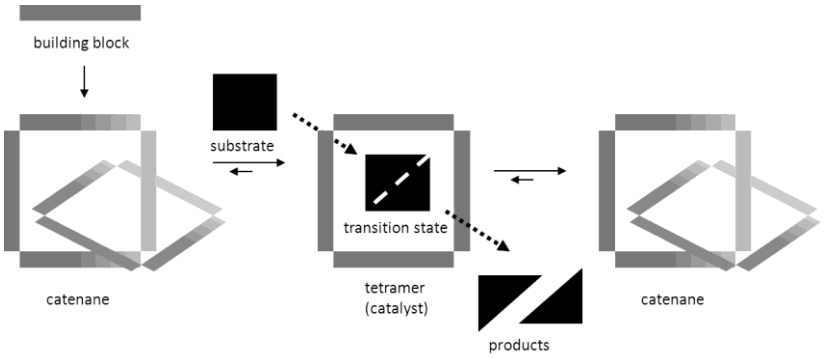


Figuur 2 Het doel: het vormen van hydrazone samenstelling met behulp van katalyse van een metal-cycleen building block welke reageert met de dialdehyde samenstelling

In Hoofdstuk 3 staan de testresultaten van het onderzoek naar disulfide dynamic combinatorial libraries. Libraries die dithiol building blocks bevatten werden verkregen, waarmee is aangetoond dat cyclen een overeenkomstige functie bevatten in disulfide libraries. Echter, door de aanwezigheid van metalen, treden er verstoringen op in de stabiliteit van de libraries. Door het veroorzaken van precipitatie problemen en veranderingen in de oxidatie snelheden van de libraries, in vergelijking tot de libraries in afwezigheid van metalen.

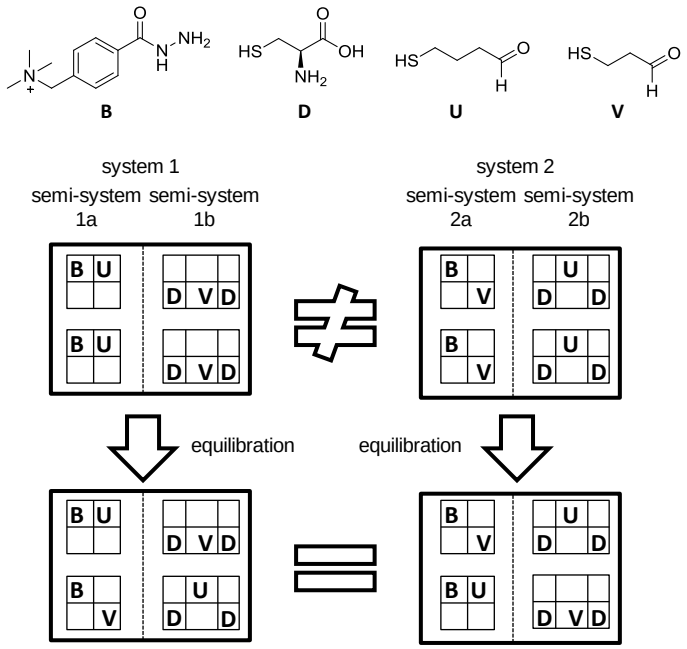
De resultaten van de dithiol katalytische libraries die in staat waren te reageren in aanwezigheid van een substraat zijn gegeven in Hoofdstuk 4. De introductie van een aza-Cope rearrangement substraat in een dynamic combinatorial library, induceerde een shift in de library compositie waarbij een katalytische soort werd gevormd die de rearrangement reactie versnelde. Nadat het substraat weg was gereageerd, veranderende de library weer in zijn begin compositie (Figuur 3). De libraries werden voorafgaand

gescreend met TSAs om de stabiliserende interacties vast te stellen, welke konden leiden tot een afname in energie van de TS van de reactie. Het werk van dit hoofdstuk bracht de eerste succesvolle toegankelijke katalytische libraries op.



Figuur 3 Een oplossing van building blocks leiding tot een dynamic combinatorial library waar de dominante library member veranderd van initieël catenane tot een katalytisch tetrameer door de additie van het reactie substraat. De DCL verschuift weer richting het initiële catenane na het consumeren van het substraat.

In Hoofdstuk 5 werd de wisselwerking van systemen van gescheiden DCL's die onderling met elkaar reageerden bestudeerd. Hydrazone libraries in waterige oplossing werden verbonden door een organische bulk, waardoor apolaire moleculen overgedragen werden en zo in staat waren zich als signalen te gedragen. De compositie van elk van deze verbonden libraries varieërde afhankelijk van de distributie van de library members van de tegengestelde library. Tevens werd er een analoog systeem ontwikkeld voor communicerende hydrazone en disulfide uitwisselingsreacties (Figuur 4). Voor het eerst werd een simultaan evenwicht van deze twee typen DCL's verkregen. Bovendien werden er preliminaire resultaten verkregen van communicerende moleculaire netwerken die waren ingesloten in liposomen. De ontwikkeling van deze chemische communicatie systemen leiden mogelijk tot het gebruik van een vereenvoudigde versie van biologische communicatie structuren.



Figuur 4 Een schematische weergave van een eerste en laatste stadium van twee initiële verschillende systemen, gevormd door een waterige hydrazone libraby en een waterige disulfide libraby, verbonden door een organisch oplosmiddel.

Acknowledgements

I arrived to Groningen with an open mind ready to spend a few years learning chemistry as well as another way of life. It was November, and the first weeks in The Netherlands nearly made me change my mind and come back to where I came from. I had my first experience with biking to work during a Dutch winter, when a single trip could include rain, snow and hail, seasoned with a strong wind. I did not have a place to stay so I spent the first month in a bed & breakfast. It was quite hard and I felt lonely and far from home. Fortunately that changed soon. I found a good place to live where I felt comfortable, I started to know people and discovered a charismatic city. Furthermore, the research was interesting and allowed me to learn a lot of things. Overall, I can say that this time in Groningen has been very enriching for my personal and professional development. I am really happy that I made the decision of doing a PhD in the Netherlands and I will always remember this period of my life.

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